Full Length Research Paper

Application of response surface methodology optimization for the production of caffeic acid from tobacco waste

Jun Wang[1], Dingqiang Lu[1,2]*, Hui Zhao[1], Xiuquan Ling[1], Ben Jiang[1] and Pingkai Ouyang[1]

[1]College of Life Science and Pharmaceutical Engineering, Nanjing University of
Technology, Nanjing 210009, P.R. China.
[2]Jiangsu Provincial Institute of Material Medica, Nanjing 210009, P.R. China.

Caffeic acid (CA) was widely applied in food and pharmaceutical industries as an important natural antioxidant. In the present study, the main caffeoylquinic acids (CQAs) in tobacco waste were analyzed by HPLC-PAD-ESI-MS/MS, and used as raw material for producing CA which preparation process was optimized by response surface methodology (RSM). The research results indicated the main ingredients of CQAs in tobacco waste were identified as three isomers containing chlorogenic acid (5-caffecylquinic acid, 5-CQA), cryptochlorogenic acid (4-caffecylquinic acid, 4-CQA) and neochlorogenic acid (3-caffecylquinic acid, 3-CQA), respectively. The production of CA produced by alkaline hydrolysis of the CQAs extract from tobacco waste was effectively enhanced by RSM. NaOH was selected as catalyst through screening of four different alkalis. Three hydrolysis reaction parameters for production of CA were optimized as follows by using central composite rotatable design (CCRD) of RSM: pH value 13.5, reaction temperature 85°C and reaction time 10 min. The adequately high R^2 value 0.9275 and F score 7.105 indicated the statistical significance of the model. The conversion rate of CA after optimization was 43.46% while the predicted value was 44.92%. In conclusion, hydrolysis conditions optimization to enhance CA production from tobacco waste can be easily and effectively done by RSM; the developed production process of CA indicated CQAs in tobacco waste are potential raw material for producing CA.

Key words: Caffeic acid, caffeoylquinic acids (CQAs), hydrolysis reaction parameter optimization, response surface methodology, tobacco waste, HPLC-PAD-ESI-MS/MS.

INTRODUCTION

Tobacco (*Nicotiana tobaccum* L.) is one of the major non-food cash crops grown in China, whose production and cultivated area has increased constantly since 1979. As new relevant information show, China produced about 1840.5 million kg tobacco leaves in 2005, which amounts to 36.5% of the total output, and China has become the largest producing country in the world (Zhu et al., 2006). Tobacco is mainly used for making cigarettes, cigars and for chewing. Large quantities of tobacoo wastes are generated during processing and cigarette making, and has become the largest tobacco wastes producing coun-

try they amount to 25% of total tobacco material. China has become the largest producing tobacoo waste country in the world. Liquid and solid wastes are a huge and always increasing problem for the industries and the environment (Michailof et al., 2008). One of the most popular ways of dealing with solid waste is its use as a raw material for the production of high additional value products.

Nowadays, the food and agricultural products processing industries generate substantial quantities of phenolic rich by-products, which could be valuable natural sources of antioxidants to be employed as ingredients. Some of these by-products have been the subject of investigation and have proven to be effective sources of phenolic antioxidants (Roldan et al., 2008). It is noteworthy that there are several biologically important com-

---

*Corresponding author. E-mail: ludingqiang@njut.edu.cn. Tel: 86-25-8328-5202.
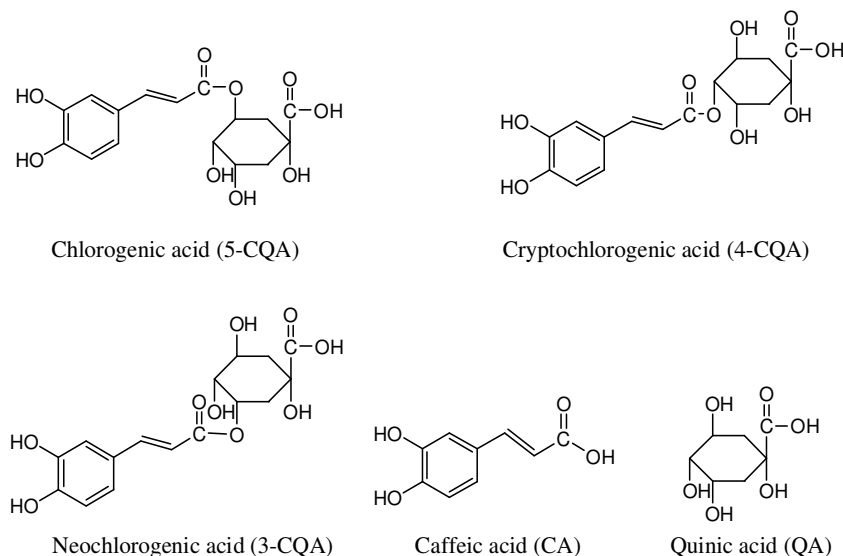
Figure 1. Structures of the chlorogenic acids and their hydrolysis products.

pounds in tobacoo waste, such as phenolic compounds (Johnson et al., 2001), solanesol (Zhao et al., 2007), proteins (Howles et al., 1996) and so on. In view of the potential pharmaceutical application, the phenolic compounds from tobacoo waste is thus of essential importance.

Chlorogonic acid (5-caffeoylquinic acid, 5-CQA) is the only one compound in the big family. The best known conjugates are those with quinic acid, collectively known as chlorogenic acids (CGAs) (Clifford et al., 2003) or caffeoylquinic acids (CQAs). Chlorogenic acids family is mainly composed of four monomers: chlorogenic acid (5-CQA), cryptochlorogenic acid (4-CQA), neochlorogenic acid (3-caffeoylquinic acid, 3-CQA) and 1-CQA (1-caffeoylquinic acid). However, they have the same hydrolysis products containing caffeic acid (CA) and D-(-)-quinic acid (QA) (Figure 1). Although the analysis method and technology of CQAs in some plant materials based on LC-MS have evolved very rapidly (Carini et al., 2001; Curadi et al., 2005; Clifford et al., 2006; Clifford et al., 2006; Bravo et al., 2007; Wang et al., 2009), few relevant report is available in hydrolysis reaction system of tobacco waste. Therefore, identification of CQAs in hydrolysis reaction system of tobacco waste is a basic research due to its high additional value application in food and pharmaceutical industry.

CA is one of the natural phenolic compounds widely distributed in plant materials such as vegetables, fruits, coffee and tea. Recently, pharmacological studies have shown that CA have antihyperglycemic (Hsu et al., 2000) , α-tocopherol protectant (Gülçin et al., 2006), antioxidative (Jiang et al., 1997; Gülçin, 2006) , anti-apoptotic (Jiang, 1997; Nardini et al., 2001; Xu et al., 2005; Khanduja et al., 2006; Pari et al., 2008), antidepressive-like (Takeda et al., 2002; Takeda et al., 2006), anti-cancer (Gao et al., 2000), and inhibits protein tyrosine kinase activity (Nardini and

Leonardi et al., 2001). On the other hand, CA interferes with the rooting potential of mung bean hypocotyl cuttings by altering the activities of peroxidases (PODs) and polyphenol oxidases (PPOs) and the endogenous total endogenous phenolics (TP) content that play a key role in rhizogenesis (Batish et al., 2008). CA electrochemical behavior on the electrode is helpful to interpret the oxidation mechanisms in the body (Hotta et al., 2002; Trabelsi et al., 2004; Moghaddam et al., 2007). Therefore, CA is an organic acid with prominent bioactivity and is used widely in the medicine, food, and cosmetic industries.

CA, produced by alkaline hydrolysis of the chlorogenic acid methanol extract, has shown the same antioxidant effectiveness as the original extract at low temperature, but it was significantly more effective at 110°C (118% compared to the control) (De Leonardis et al., 2005). So, production of CA by alkaline hydrolysis of the chlorogenic acids from tobacco waste is a good way to provide natural antioxidants. At the same time, processing and stabilizing tobacco wastes could represent both advantages: a solution of the environmental problem derived from a great tobacco wastes disposal and production of stabilized tobacco by-products as natural antioxidant food ingredients.

The limitations of classical method of reaction parameters optimization can be overcome by the application of statistical based approach. RSM, an extensively used statistical technique for media optimization, is a collection of statistical techniques which uses design of experiments (DoE) for building models, evaluating the effects of factors and searching for the optimum conditions (Liyana-Pathirana et al., 2005; Rodr et al., 2007; Rold et al., 2008) . To the best of our knowledge, up to now, there is no information in the literature on preparation processing study of CA by alkaline hydrolysis of the chlorogenic acids

from tobacco waste. Therefore, in the present work, we have used a central composite rotatable design (CCRD) of response surface methodology for reaction conditions optimization to enhance CA production from tobacco waste.

MATERIALS AND METHODS

Plant materials

Tobacoo wastes were collected in September 2007, at Jiangsu Tobacco Company Nanjing Branch in China, and authenticated as the wastes of *N. tobaccum* L. by Prof. Dingqiang Lu (College of Life Science and Pharmaceutical Engineering, Nanjing University of Technology). The sample was dried at 60°C, powered by a herb disintegrator (Qinzhou Sanyang Package Equipment Co., Ltd) and then sieved (60 mesh).

Chemicals and reagents

HPLC-grade acetonitrile and acetic acid were purchased from TEDIA Co. (Fairfield ,OH, USA); Chlorogenic acid (5-caffecylquinic acid, 5-CQA) and caffeic acid (CA) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), Beijing, China. Cryptochlorogenic acid (4-CQA) and neochlorogenic acid (3-CQA) were purchased from Chendu Biopurify Phytochemicals Ltd. (Chendu, China).

The reference standards of the target compounds, i. e., neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid and caffeic acid were accurately weighted and dissolved in methanol, then diluted to appropriate concentration ranges for the establishment of calibration curves. All solutions were stored at 4°C.

Analytical methods

HPLC-PAD-MS/MS was carried out on a Waters system (Millipore Corp., Milford, MA, USA). The LC equipment comprised a Waters 2695 Separations Module, autosampler with 50 μL loop, and a Waters 2996 Photodiode Array Detector with a light-pipe flow cell (recording at 328, 320, 280, and 254 nm, and scanning from 200 to 400 nm). This was interfaced with a mass spectrometer fitted with MICROMASS® Quattro micro ™ API source and ESCi ™ MULTI-MODE IONIZATION Plus ESI source.

HPLC-PAD-ESI-MS/MS experiment was performed according to reference literature (Wang et al., 2008 and 2009). HPLC separation was performed on an Alltima C_{18} (250×4.6 mm, 5 µm) column (Alltech, Deerfield, IL, USA). The mobile phase contained solvents A and B, where A was water : acetonitrile : acetic acid (97.5:2:0.5, v/v/v) and B was acetonitrile : acetic acid (99.5:0.5, v/v). The linear gradient profile was maintained at 2% B in 5 min, from 2 to 5% in 5 min, from 5 to 10% in 20 min, from 10 to 35% in 35 min, from 35 to 100% in 15 min, from 100 to 2% in 5 min, and maintained at 2% B in 5 min. The wavelength range of PAD detection was from 200 to 400 nm. The flow-rate was 1 mL/min for HPLC and PAD detection with the column kept at 30°C. A splitter was connected between the PAD and MS detectors, which reduced the flow rate to 0.2 mL/min for MS detection. The electrospray ESI-MS was operated in the negative ion mode with fragmentation amplitude 1.5, scanning range of m/z 100-800 and capillary voltage of 3.0 kV. High purity nitrogen (99.9%) was used as dry gas to evaporate the solvent at a flow rate of 500 mL/min, temperature of 350°C. Nitrogen was used as nebuliser gas at 50 psi.

HPLC-UV separation was performed on an Alltima C18 (250 × 4.6 mm, 5 µm) column (Alltech, Deerfield, IL, USA). The mobile   phase

contained acetonitrile: ammonium acetate buffer (pH 4.5) (5:95, v/v). The flow-rate was 1 mL min^{-1} and UV detection with the column kept at 30°C, detected at 327nm. All solutions were filtered through a 0.45 µm filter before injection. All samples were determined in triplicate (Wang et al., 2009).

Hydrolysis experiments

The hydrolysis experiments were carried out in a three nick round flask and heated in a temperature controlled heating water bath (Jiangsu Taicang Experimental Instrumental Factory, China). Various alkalis (KOH, NaOH, Na_2CO_3 and $NaHCO_3$) were performed to catalyze hydrolysis reaction for 30 min at 75°C, and then the optimal alkali was selected by determining the conversion rate of CA in different catalysis reaction system. Following that, appropriate range of hydrolysis reaction temperature and time were performed by Allen' s method.

Experimental design and optimization by response surface methodology (RSM)

The initial assays were based in a 3^3 factorial design with three different pH values, as well as three different hydrolysis reaction temperatures and times. The composition of the model was established from these preliminary assays. The model composition corresponded to an orthogonal 3^3 design, following the methodology of Box-Behnken response surface design (Anunziata et al., 2008; Balderas Hernandez et al., 2008). For statistical calculation, the variables were coded according to (Canettieri et al., 2007). The statistical model was based on the RSM which equation was determined by analysis of linear multiple regression using the software STATISITICA 6.0 (Statsoft, USA). The conversion of CA produced by alkaline hydrolysis of the CQAs from tobacco wastes was taken as the dependent variable or response of the design experiments. The statistical significance of the regression coefficients was determined by T test. The variables were correlated by empirical models.

Conversion rate of CA (%)

The conversion rate of CA was calculated as expressed in Eq. (1).

$$\text{Conversion rate of CA (\%)} = \frac{\text{molar concentration of CA (mol/L)}}{\text{molar concentration of CQAs (mol/L)}} \times 100\% \qquad (1)$$

where molar concentration of CQAs is the sum of the molar concentration of 3-CQA, 4-CQA and 5-CQA in material sample of tobacco wastes.

RESULTS AND DISCUSSION

Composition of CQAs in tobacco wastes

Figure 2 shows the HPLC Chromatographic profiles of the 70% ethanol extract of tobacco waste detected with negative ion mode ESI-MS/MS and UV at 320 nm.

In the methanolic extract of dried tobacoo waste, 3-CQA, 4-CQA and 5-CQA were identified by comparing their retention times, UV and MS spectra with those of standard compounds.

The structure of 3-CQA, 4-CQA and 5-CQA were determined by MS SCAN model, and by comparing the UV and MS spectra data with those reported in   literature
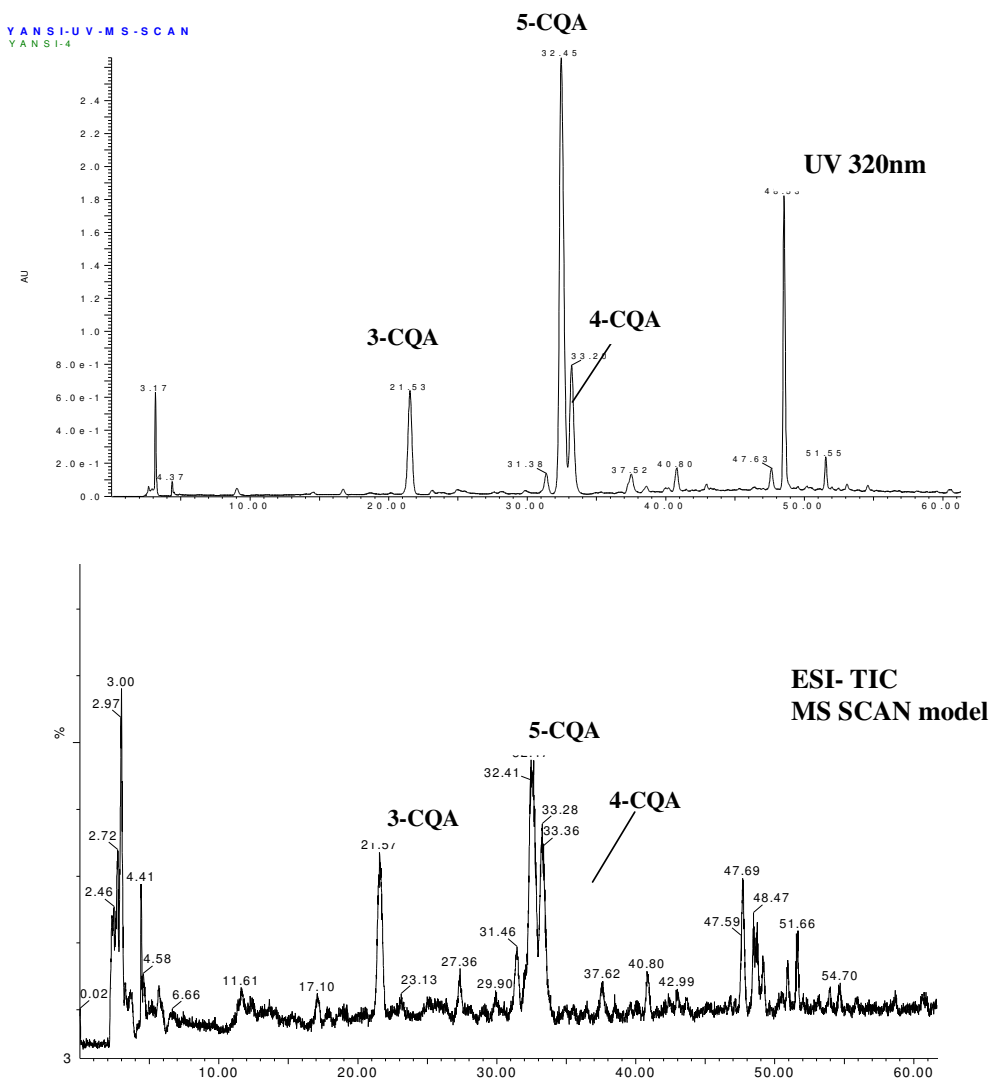
Figure 2. Chromatographic profiles of a methanolic extract of tobacoo wastes.

(Wang et al., 2009; Clifford et al., 2003 and 2006). These results show that CQAs in tobacco waste is mainly composed of three monomers: 3-CQA, 4-CQA and 5-CQA.

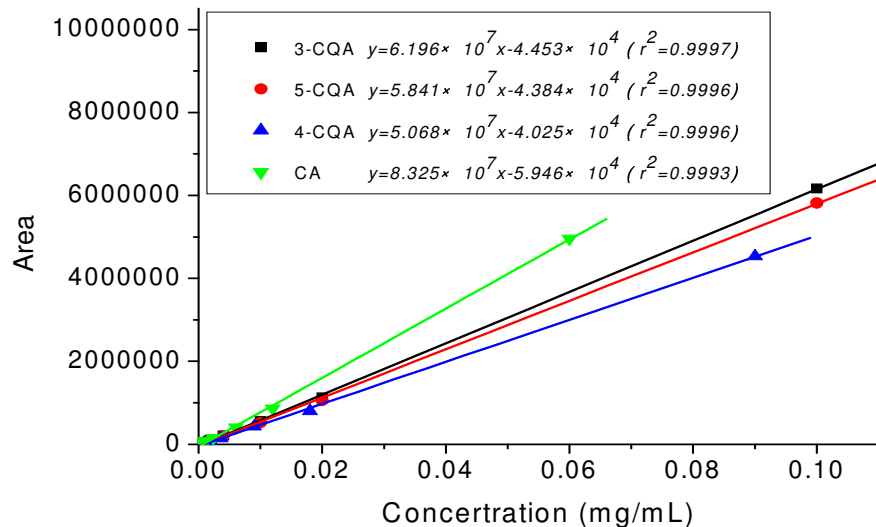## Calibration data for 3-CQA, 5-CQA, 4-CQA and CA

Peaks of CQAs and CA can be well separated from all the other components in tobacco waste. Each component in the samples analyzed was identified by comparing its retention time with that of respective standard. Retention time for 3-CQA, 5-CQA, 4-CQA and CA were 4.9, 6.2, 7.4 and 11.3 min, respectively. Quantification was carried out by integration of the peaks using external standards. The results of Calibration data for 3-CQA, 5-CQA, 4-CQA and CA are shown in Figure 3, which indicated that the developed analytical method is precise and sensitive for determining four components in test samples (Wang et al.,

2009).

Selection of optimum catalyst, pH value, reaction temperature and time for producing CA from tobacco waste

The degradation of CQAs was mainly a chemical process achieved by hydrolysis of ester bond. The results indicated that four alkalis (KOH, NaOH, Na_2CO_3 and $NaHCO_3$) showed certain catalytic activities for producing CA from tobacco waste (Figure 4). Compared with the others, NaOH exhibits higher catalytic activity for hydrolysis of the CQAs extract from tobacco waste. This indicates that NaOH is a suitable alkaline that can be used for catalytic hydrolysis of the CQAs extract from tobacco waste.
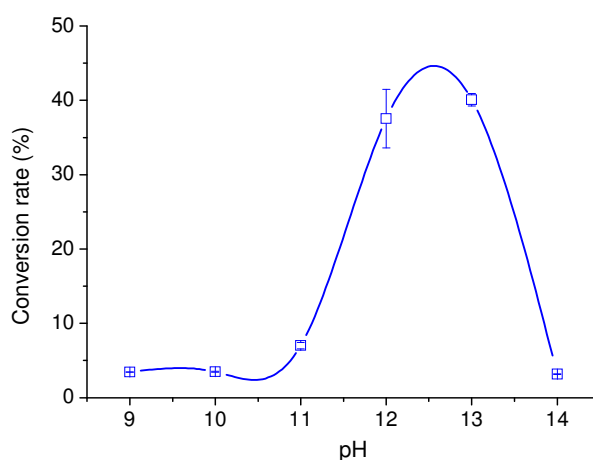
The conversion rate of CA increased with pH from 9 to

Figure 3. Calibration data for 3-CQA, 5-CQA, 4-CQA and CA (n=6).
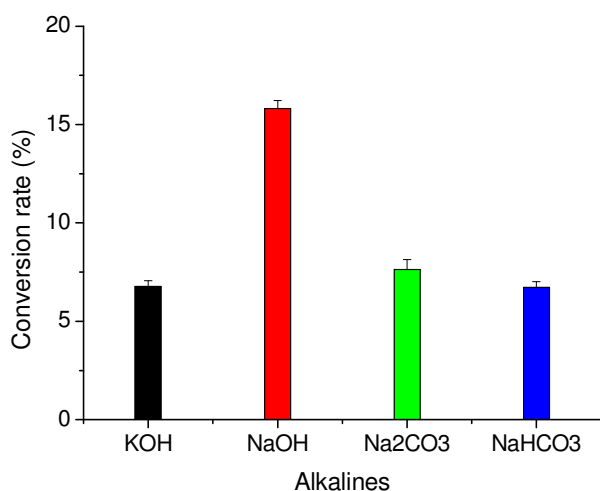
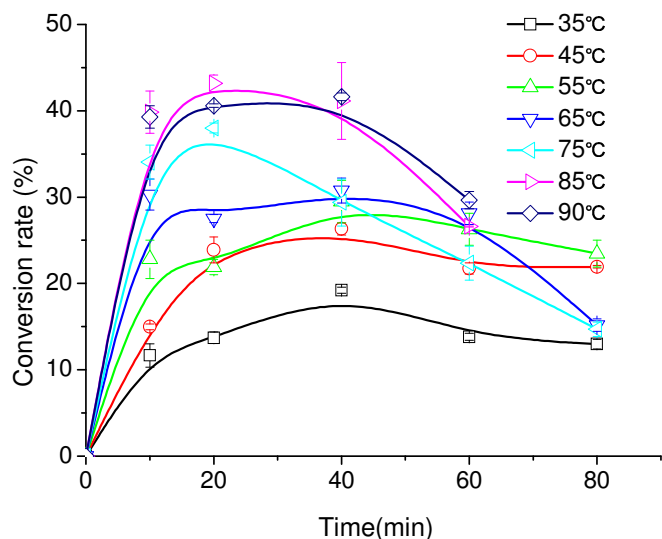**Figure 4.** The influence of different catalyst during CA conversion from tobacco waste.

Figure 5. The influence of different pH during CA conversion from tobacco waste.

13, and thereafter decreases between pH 13 to 14 (Figure 5). Therefore, 12~13 was selected the most suitable pH value for the production of CA from tobacco waste.

The concentration of hydrolysis reaction temperature and time influence the conversion rate of CA produced by NaOH hydrolysis of CQAs extract from tobacco waste (Figure 6). The highest conversion rate of CA is at the hydrolysis reaction temperature of 85°C and time of 20 min. The curves of CA conversion rate between times at various temperatures indicated that hydrolysis temperature and time are two important parameters in process of production of CA. For the different reaction temperatures (35, 45, 55, 65, 75, 85 and 90°C), the conversion rate of CA increased with reaction time, and reached the maximal value when the hydrolysis time were 40, 40, 40,

40, 20, 20 and 20 min, respectively. And then, the conversion rate of CA decreased with the increase in the hydrolysis time. It can also be seen from Figure 6, within the temperatures investigated, at the same hydrolysis time such as 20 min, the conversion rate of CA increased with the increasing temperature; the conversion speed increased with the increasing temperature, too. However, with the increase of reaction time, the degradation speed of CA is increases, so selecting optimum hydrolysis temperature and time is essential to obtain maximum conversion rate of CA. In the comprehensive consideration of the hydrolysis temperature and time, 65~85°C and 10~40 min were selected the most suitable hydrolysis temperature and time, respectively, for the production of CA from tobacco waste and were used in the subsequent assays.

Figure 6. The influence of different temperature during CA conversion from tobacco waste.

Optimization of alkaline hydrolysis and validation of statistical model

The coded values of independent variables are given in Table 1. The design of experiments through alkaline hydrolysis and the respective experimental and predicted conversion rate of CA from CQAs in tobacco waste are given in Table 2. The results on estimated effects, standard errors (SE), t test and significance level for the model representing CA conversion rate from the CQAs in tobacco waste are presented in Table 2.

Data were analyzed by non-linear multiple regression using STATISTICA software (Statsoft, v. 6.0). After regression analysis, the second-order response model was obtained which is given in Eq. (2).

$$\text{Conversion rate of CA (\%)} = 12.965X_1 - 9.109X_1^2 + 6.672X_2 - 4.406X_2^2 \\ - 0.021X_3 - 0.626X_3^2 + 1.472X_1X_2 - 3.789X_1X_3 \\ - 2.233X_2X_3 + 31.931 \tag{2}$$

The simple model expressed by Eq. (3) was generated as:

$$\text{Conversion of CA}_{)=} 12.965X_1 - 9.109X_1^2 + 6.672X_2 + 31.931 \tag{3}$$

A low value of the coefficient of variation indicates the very high degree of precision and a good reliability of the experimental values. The fit of the model was also expressed by the coefficient of determination R^2 (F score 7.105), which was found to be 0.9275, indicating that 92.75% of the variability in the response could be explained by the model. The solution was obtained by submitting the levels of the factors into the regression equation.

Table 1. Coded values of independent variables.

No.	Variables	Coded values		
		-1	0	1
1	pH	11.5	12.5	13.5
2	Temperature (°C)	55	70	85
3	Time (min)	10	25	40

To determine the most adequate operating conditions and to analyze the process of hydrolysis reaction, the response surfaces were plotted using Eq. (2) for three possible combinations. The response surface and contour diagrams of conversion rate of CA as a function of: (a) X_1 and X_2, (b) X_1 and X_3, (c) X_2 and X_3 are presented in Figure 6. The simultaneous analysis of so many plots is a complex task if practical short cuts taking advantage of prior knowledge of the process are not adopted. In Figure 7, the main factors effects conversion rate of CA produced by alkaline hydrolysis of CQAs from tobacco waste were pH value and hydrolysis reaction temperature. For example, from Figure 7 it can be seen that low temperature and pH value in the process of hydrolysis reaction lead to a low conversion rate of CA, so the pH value and hydrolysis reaction temperature can be selected at an appropriate value range.

The optimum values were found by solving the regression equation analytically (Agarry et al., 2008). The solution was obtained by submitting the levels of the factors into the regression equation (Eq. 2). The optimal hydrolysis reaction conditions for the production of CA from tobacco wastes were calculated as followed: pH 13.5, temperature, 85°C and time 10 min. The predicted response (44.92%) was experimentally verified (43.46%, n=3). The agreement between predicted value and experimental value of conversion rate of CA confirms the significance of the model. This indicated that, in addition to establishing optimal conditions for operation, the present methodology also makes it possible to predict conversion rate when the system is disturbed in some way. This is useful not only for the additional knowledge supplied about the process, but also for the potentials for process control (Kalil et al., 2000). Therefore, under optimal conditions of hydrolysis reaction, it is inevitable to have a large increase in conversion rate of CA.

Conclusions

Three CQAs (3-CQA, 4-CQA and 5-CQA) in tobacco wastes were identified by HPLC-PAD-ESI-MS/MS, and the optimal production process of CA by alkaline hydrolysis of CQAs in methanol extract from tobacco wastes was successfully achieved by RSM in this study. Three hydrolysis reaction parameters for production of CA were optimized as follow by using CCRD of RSM: pH

Table 2. Response surface methodology (RSM) design of independent variables and their corresponding experimental and predicted conversion rate of CA from tobacco wastes after alkaline hydrolysis.

No.	X_1 (pH)	X_2 (Temperature)	X_3 (Time)	Conversion rate (%)	Conversion rate (%) Predicted
1	-1	-1	0	5.078	0.252
2	1	-1	0	25.841	23.237
3	-1	1	0	8.048	10.652
4	1	1	0	34.699	39.525
5	-1	0	-1	6.695	5.463
6	1	0	-1	42.424	38.970
7	-1	0	1	9.546	13.000
8	1	0	1	30.119	31.351
9	0	-1	-1	11.958	18.015
10	0	1	-1	37.197	35.825
11	0	-1	1	21.068	22.440
12	0	1	1	37.275	31.317
13	0	0	0	31.455	31.931
14	0	0	0	30.915	31.931
15	0	0	0	33.424	31.931

*Values indicate mean of dupilicate observations.

(a)

(b)
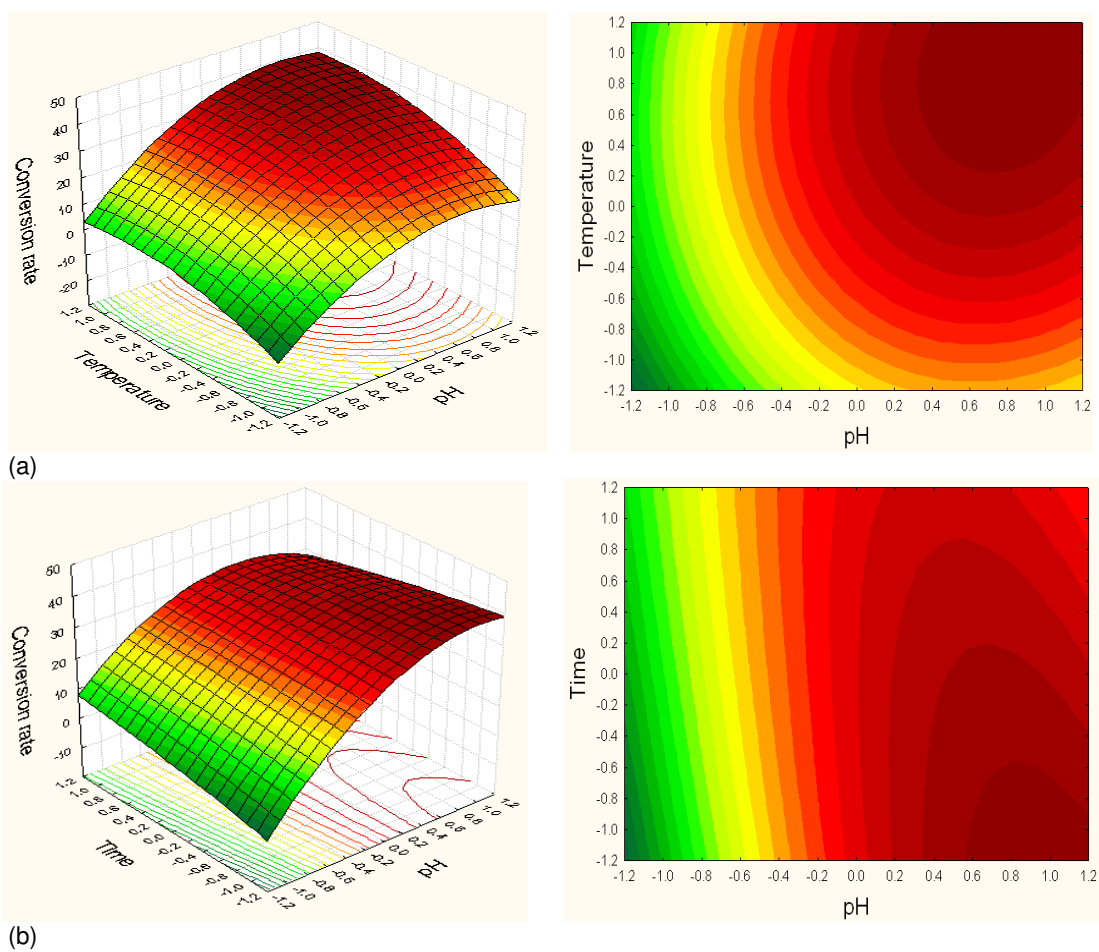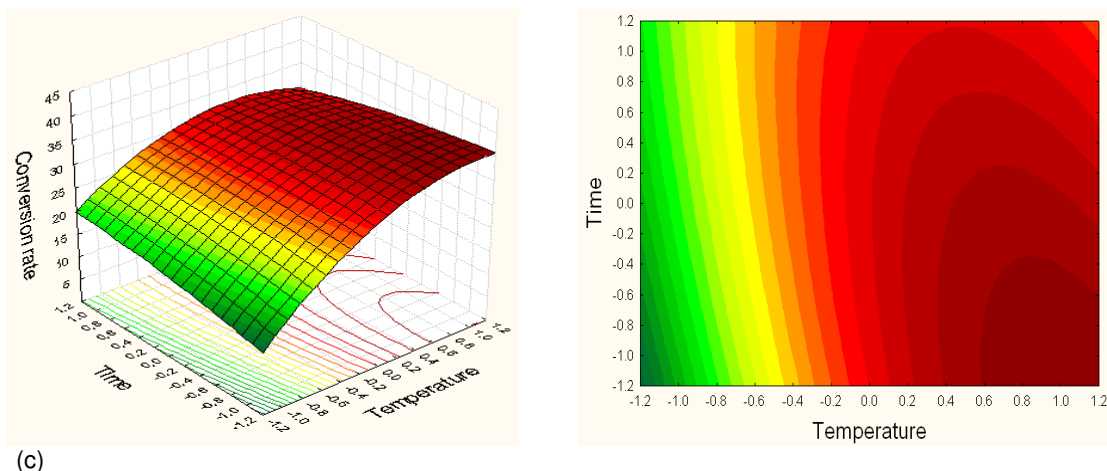
Figure 7. Response surface and contour diagrams of conversion rates of CA as a function of (a) X_1 and X_2, (b)X_1 and X_3, (c) X_2 and X_3.

value 13.5, reaction temperature 85°C and reaction time 10 min. The conversion rate of caffeic acid after optimization was 43.46% while predicted value, 44.92%. RSM as an effective method proved to be a quite adequate for the design and optimization of the process of hydrolysis reaction of CQAs from tobacco waste, and the developed production process of CA indicated CQAs in tobacco waste are potential raw material for producing CA.

ACKNOWLEDGEMENTS

REFERENCES

Agarry SE, Solomon BO, Layokun SK (2008). Optimization of process variables for the microbial degradation of phenol by Pseudomonas aeruginosa using response surface methodology. Afr. J. Biotechnol. 7(14): 2409-2416.

Anunziata OA, Cussa J (2008). Applying response surface design to the optimization of methane activation with ethane over Zn-H-ZSM-11 zeolite. Chem. Engl. J. (Amsterdam, Neth.) 138 (1-3): 510-516.

Balderas Hernandez VE, Paz Maldonado LMT, Rivero EM, Barba de la Rosa AP, Ordonez Acevedo LG, De Leon Rodriguez A (2008). Optimization of human interferon gamma production in Escherichia coli by response surface methodology. Biotechnol Bioprocess Eng. 13 (1): 7-13.

Bravo L, Goya L, Lecumberri E (2007). LC/MS characterization of phenolic constituents of mate (Ilex paraguariensis, St. Hil.) and its antioxidant activity compared to commonly consumed beverages. Food Res. Int. 40 (3): 393-405.

Canettieri EV, Rocha GJDM, de Carvalho JA, Silva JBDAE (2007). Optimization of acid hydrolysis from the hemicellulosic fraction of Eucalyptus grandis residue using response surface methodology. Bioresource Technol. 98 (2): 422-428.

Carini M, Aldini G, Furlanetto S, Stefani R, Facino RM (2001). LC coupled to ion-trap MS for the rapid screening and detection of polyphenol antioxidants from *Helichrysum stoechas*. J. Pharm. Biomed. Anal. 24(3): 517-526.

Clifford MN, Johnston KL, Knight S, Kuhnert N (2003). Hierarchical scheme for LC-MSn identification of chlorogenic acids. J. Agric. Food Chem 51: 2900-2911.

Clifford MN, Knight S, Surucu B, Kuhnert N (2006). Characterization by LC-MSn of Four New Classes of Chlorogenic Acids in Green Coffee Beans: Dimethoxycinnamoylquinic Acids, Diferuloylquinic Acids, Caffeoyl-dimethoxycinnamoylquinic Acids, and Feruloyl-dimethoxycinnamoylquinic Acids. J. Agric. Food Chem. 54(6): 1957-1969.

Clifford MN, Marks S, Knight S, Kuhnert N (2006). Characterization by LC-MSn of Four New Classes of p-Coumaric Acid-Containing Diacyl Chlorogenic Acids in Green Coffee Beans. J. Agric. Food Chem. 54(12): 4095-4101.

Curadi M, Ceccarelli N, Picciarelli P, Graifenberg A (2005). Quali-quantitative determination of chlorogenic acid in artichoke heads by RP-HPLC and GC/MS. Acta Horticulturae 681 (Proceedings of the Fourth International Congress on Artichoke, 2000): 511-515.

De Leonardis A, Macciola V, Di Domenico N (2005). A first pilot study to produce a food antioxidant from sunflower seed shells (Helianthus annuus). Eur J Lipid Sci Technol 107 (4): 220-227.

Gülçin I (2006). Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). Toxicology 217 (2-3): 213-220.

Gao T, Ci Y, Jian H, An C (2000). FTIR investigation of the interaction of tumor cells treated with caffeic acid and chlorogenic acid. Vib Spectrosc 24 (2): 225-231.

Hotta H, Ueda M, Nagano S, Tsujino Y, Koyama J, Osakai T (2002). Mechanistic Study of the Oxidation of Caffeic Acid by Digital Simulation of Cyclic Voltammograms. Anal. Biochem. 303 (1): 66-72.

Howles PA, Sewalt VJH, Paiva NL, Elkind Y, Bate NJ, Lamb C, Dixon RA (1996). Overexpression of L-Phenylalanine Ammonia-Lyase in Transgenic Tobacco Plants Reveals Control Points for Flux into Phenylpropanoid Biosynthesis. Plant Physiol. 112: 1617-1624.

Hsu L, Chen YC, Cheng JT (2000). Caffeic acid as active principle from the fruit of Xanthium strumarium to lower plasma glucose in diabetic rats. Planta-Med. 66 (3): 228-230.

Jiang Hong Chen CH (1997). Antioxidant Activities of Caffeic Acid and Its Related Hydroxycinnamic Acid Compounds. J. Agric. Food Chem. 45(7): 2374-2378.

Johnson KS, Felton GW (2001). Plant phenolics as dietary antioxidants for herbivorous insects: a test with genetically modified tobacco. J. Chem. Ecol. 27(12): 2579-2597.

Kalil SJ, Maugeri F, Rodrigues MI (2000). Response surface analysis and simulation as a tool for bioprocess design and optimization. Process Biochem. (Oxford) 35(6): 539-550.

Khanduja KL, Avti PK, Kumar S, Mittal N, Sohi KK, Pathak CM (2006). Anti-apoptotic activity of caffeic acid, ellagic acid and ferulic acid in

normal human peripheral blood mononuclear cells: a Bcl-2 independent mechanism. Biochem. Biophys. Acta. 1760(2): 283-289.

Liyana-Pathirana C, Shahidi F (2005). Optimization of extraction of phenolic compounds from wheat using response surface methodology. Food Chem. 93(1): 47-56.

Michailof C, Stavropoulos GG, Panayiotou C (2008). Enhanced adsorption of phenolic compounds, commonly encountered in olive mill wastewaters, on olive husk derived activated carbons. Bioresour. Technol. 99(14): 6400-6408.

Moghaddam AB, Ganjali MR, Dinarvand R, Norouzi P, Saboury, AA Moosavi-Movahedi AA (2007). Electrochemical behavior of caffeic acid at single-walled carbon nanotube:graphite-based electrode. Biophys. Chem. 128(1): 30-37.

Nardini M, Leonardi F, Scaccini C, Virgili F (2001). Modulation of ceramide-induced NF-[kappa]B binding activity and apoptotic response by caffeic acid in U937 cells: comparison with other antioxidants. Free Radical Bio. Med 30(7): 722-733.

Pari L, Prasath A (2008). Efficacy of caffeic acid in preventing nickel induced oxidative damage in liver of rats. Chem. Bio. Int. 173(2): 77-83.

Rodriguez-Nogales JM, Ortega N, Perez-Mateos M, Busto MD (2007). Experimental design and response surface modeling applied for the optimisation of pectin hydrolysis by enzymes from A. niger CECT 2088. Food Chem 101 (2): 634-642.

Roldán E, Sánchez-Moreno C, de Ancos B, Cano MP (2008). Characterisation of onion (Allium cepa L.) by-products as food ingredients with antioxidant and antibrowning properties. Food Chem. 108 (3): 907-916.

Takeda H, Tsuji M, Inazu M, Egashira T, Matsumiya T (2002). Rosmarinic acid and caffeic acid produce antidepressive-like effect in the forced swimming test in mice. Eur. J. Pharm. 449(3): 261-267.

Takeda H, Tsuji M, Yamada T, Masuya J, Matsushita K, Tahara M, Iimori M, Matsumiya T (2006). Caffeic acid attenuates the decrease in cortical BDNF mRNA expression induced by exposure to forced swimming stress in mice. Eur. J. Pharm. 534(1-3): 115-121.

Trabelsi SK, Tahar NB, Abdelhedi R (2004). Electrochemical behavior of caffeic acid. Electrochimica Acta 49 (9-10): 1647-1654.

Wang J, Lu DQ, Qiao HQ, Ling XQ, Zhao H, Ouyang PK (2008). HPLC-PAD-ESI/MS/

Xu JW, IkedaK, Kobayakawa A, Ikami T, Kayano Y, Mitani T, Yamori Y (2005). Downregulation of Rac1 activation by caffeic acid in aortic smooth muscle cells. Life Sci. 76(24): 2861-2872.

Zhao Y, Du Q (2007). Separation of solanesol in tobacco leaves extract by slow rotary counter-current chromatography using a novel non-aqueous two-phase solvent system. J. Chromatogr. A 1151 (1-2): 193-196.

Zhu X, Zhu X, Zheng F, Cao Z (2006). World tobacco production report in 2005. Acta Tabacaria Sin. 12(4): 58-64.