Solvent and Supercritical Fluid Extraction of Catechin from *Camellia Sinensis* (tea) Leaves for Utilization as Functional Food Ingredient

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***Abstract*-- Supercritical fluid and solvent extraction of catechin from green tea (*Camellia sinensis)* leaves was studied. Response surface methodology was used to design the experiment and optimize the operating variables (pressure, temperature and dynamic time). The optimum recovery of the extractable crude yield (6.65%), TPC (28.07g/100gGAE), EGCG (263.06mg/100ml), and total catechin (844.25mg/100ml) were obtained at pressure (30Mpa), temperature (47.99^{o}C and dynamic time(40 minutes) with a co-solvent (50% v/v water :ethanol) and CO_2flow rates of 1,5 and 1.0ml/ minute respectively. When organic solvent was used, the extractable components were in the range of: total crude extract (2.05 - 3.57%), TPC (153.22 -94.95g/(100gGAE), and TC (285.27mg/100ml) respectively. (With methanol 70 % (v/v), ethanol 50% (v/v), and ethanol 70% (v/v) being the most effective in term of extracts. The result of the extracts obtained by SFE extraction when compared to that of solvent shows that SFE was more effective in the recovery of catechin polyphenols from green tea leaves. By comparing conventional solvent and SFE extraction we were able to identify the abundance of green tea polyphenol from Malaysia which can compete favourably with those obtained from other tea producing countries. The knowledge can be useful to functional food manufacturers.**

***Index Term*-- Extraction, Supercritical Fluid extraction (SFE), solvent extraction, green tea catechin and functional food.**

1.0 INTRODUCTION

Tea has its origin from China several thousand years ago during era of Shen-Nong dynasty (Zuo, Chen, & Deng, 2002). Tea is a popular drink second to water in consumption globally, most especially in East Asian countries. The global production and consumption of tea was expected to rise by 1.7% and 1.3% respectively with demands estimated at over RM54 billion by 2015 globally, (Bernama, 2007) . This presents good opportunities for tea producing countries like Malaysia, where there is limited data on the quality of teas produced. Green tea has functional properties which have been attributed to the presence of polyphenols for example catechins including; epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG) and epicatechingallate (ECG). EGCG is the most bioactive and predominant catechin compound in green tea which forms over 45% of the total catechin (Hara, 2001)

Polyphenols have been recovered from different plant materials using different extraction methods (Abascal, Ganora, & Yarnell, 2005; Dai & Mumper, 2010).

Plant samples contain a wide variety of other materials apart from phenolic acids, including anthocyanins, tannins, carbohydrates, proteins, sugars, organic acids and fats in various proportions in the extracts which may require additional steps to remove because they may not be desired (Dai & Mumper, 2010). Presently, there is no universal extraction procedure suitable for extraction of all plants phenolics to accommodate the diversity of plants materials used as source of phenolic compounds (Dai & Mumper, 2010).

Organic solvents such as methanol, ethanol, acetone ethyl acetate, have been used for the extraction of phenolic compounds in the past (Perva-Uzunalić et al., 2006). However, the use of organic solvents are not encouraged for use in the food industry due to its adverse effects on health and the environment (Dai & Mumper, 2010; Vuong, et al.; 2010).

Alternative option microwave assisted, ultrasonic-assisted and supercritical fluid extraction methods have been report (Liazid, Palma, Brigui, & Barroso, 2007). Supercritical fluid extraction (SFE) is one of the green extraction techniques which has been used as alternative to organic solvent for extraction of bioactive compounds and are considered more valuable because of the numerous advantages over the traditional solvent method (Ibanez et al.;, 2012; Roberto, Junior, Leite, Romanelli, & Dragano, 2010) SFE is most suitable for extracting heat sensitive phenolic compounds and there is little fear of residual organic compounds in the extracts (Ibañez, et al.; 2012).

There are many studies on extraction of green tea,(Ghoreishi & Heidari, 2013; Vuong, Golding, Nguyen, & Roach, 2013), there are no studies using supercritical fluid extraction for green tea leaves from Cameron highland, Malaysia. Reports have shown that cultivation practices, climatic, genetic and extraction conditions affect major bioactive polyphenols including catechins(Song, Kelman, Johns, & Wright, 2012).

The objective of this study was to optimize the extraction process of green tea (Camellia *sinensis*) from Cameron highlands and to compare the extractable yields using supercritical fluid carbon dioxide (SC-CO_2) and conventional organic solvent techniques. The roles of pressure, temperature, and dynamic time were evaluated using response surface methodology, while keeping other factors constant.

2.0 Material and methods

Healthy and fresh tea (*Camellia sinensis*) leaves composed of complete buds and other first two fully expanded leaves underneath were manually collected between June and December, 2013 in a well-aerated cool container from BOH tea plantation Cameron highlands, Malaysia, and were taken to the laboratory within 5 hours after collection. Carbon dioxide food grade liquefied (99.99% purity) was purchase from MOX-LINDE Gases Sdn, Bhd- PJ Selangor Malaysia. All solvents (Fishers) used for our analysis were of analytical or HPLC grade purchase from agents in Malaysia (Sinar Scientific, Taman Sri Serdang). Catechins reference standards (Sigma Aldrich), Folin-Ciocalteu phenol reagent Gallic acid, anhydrous sodium carbonate (Fluka), were purchased from, Sceinfield (Expert PLT Sdn, Bhd, Malaysia).

2.1 Solvent

The International Organization for standardization (ISO) 1405-1 was adopted (ISO 14502-1: 2005).The following solvents methanol, ethanol (100%, 70%, and 50% v/v) and water were used at a temperature and time of 70oC and 10minutes respectively. Extraction mixtures of 0.2g green tea powder in 20mL (10: 1 solvent/ solid ratio) were extracted in 10mL test tube with constant stirring. The extracts were centrifuged and filtered after cooling at room temperature. The supernatant was partitioned with chloroform twice. The upper layer was collected and impurities were discarded. The water layer was again partitioned with ethyl acetate twice. Catechin extract/ethyl acetate layer was collected and the water layer was discarded. The supernatant was then evaporated using rotary evaporator at 40^{o}C to obtain a semi solid extract. This was transferred into a pre-weighed watch glass and was dried at 50^{o}C for 18 hours before the percentage extract yield was determined. Some portion of the extracts was vacuumed filtered using 0.45µm and stored at -21^{o}C until during analysis.

2.2 Determination of total phenolic (TPC)

The method used by International Organization for standardization ISO14502-1(2005) as reported by (Anesini, Ferraro, & Filip, 2008) was adopted. The total phenolic content (TPC) was expressed as gallic acid equivalent (GAE g/100g) of the green tea leaves. The concentration of polyphenols in the samples was derived from a standard curve of Gallic acid of 10-50µg/ml concentration (Pearson's correlation coefficient R^2 =0.9996 -0.9899).

2.3 Determination of 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The antioxidant capacity was determined by the scavenging capacity of the DPPH based on the method used by Chen and Ho (1995), with slight modification. Briefly, 0.2mL of the green tea leave extract was added 3.8mL of ethanol solution 0.1mM DPPH solution. The mixture was vortexed for 1minute and incubated for 30 minutes at room temperature in the dark. The absorbance of the sample (A_S) and the absorbance of ethanol blank (A_B) was measured at 517nm.The DPPH scavenging activity the sample were calculated using the formula:

Percentage discolouration = $[(A_B - A_S/A_B)] \times 100$.

Free radical capacity of green tea leaves powder was expressed as an equivalent of trolox. Linearity of calibration curve was 20 to 1000mM

2.4 SFE extraction procedures of green tea leave powder

The method of Ghoreishi and Heidari (2013), was used for the extraction of green tea leaves powder using a mini laboratory proto-type supercritical fluid extractor (SFE) system (Thar Model, 500.Thar Technologies Inc., Pittsburgh, PA USA) . A 40g wt. % ground green tea powder and 60g wt. % broken Pyrex glass beads (mesh size 35) was extracted using a static time of 25minutes. The mass flow rate of CO_2 and the co-solvents were set constant at 1.5 and 1.0mL/min respectively.

2.5 Optimizing the extraction conditions of green tea leaves using central composite (CCD)

The extraction was performed using 50% ethanol as a co solvent ant SC-CO_2 (99.9%) at temperature of 40, 50 and 60^{0}C, pressure at 10, 20, and 30Mpa and dynamic time of 40, 80 and 120 minutes with a static time of 25 minutes. A factorial design of 2^2 employing the response surface methodology (RSM) using a central composite rotatable design (CCRD) with 20 experiments for the independent variables were studied with six centre points.

2.6 Determination of crude extract yield

The crude extracts collected from the traditional solvent extraction and the supercritical fluid extraction were determined after removing the residual solvent by drying in an oven at 40^{o}C for 2hours. The weight was taken at 30 minutes interval during drying until a constant weight was obtained. The dried extracts were transferred into desiccator for cooling in a dark environment. The yield was determined using the equation below:

$Y\ (\%) = (M_o \div M_1) \times 100$

Where: Y - represents percentage crude extract yield (%), M_o (g) - is mass of crude extract (g) and, M_1 - is the feed mass (g).

2.7 Experimental design, regression modelling and statistical analysis

. A factorial design of 2^2 employing the response surface methodology (RSM) using a central composite design (CCD) with 20 experiments with six centre points was used in the collection of data using three independent variables (pressure (10, 20,and 30Mpa), temperature (40,50 and 60^{o}C) and time (40,80 and120 minutes) at three levels as presented in **Table1**. The results from the experimental data were interpreted to enable us know the degree of the variation of Y (dependent variables) on X (independent variables) using second-order polynomial model equation used for response surface model as expressed below:

$$Y= \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X^2{}_i + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ii} x_i x_j \quad (1)$$

Where Y is the response, β_o the constant coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} represent the interaction coefficient while X_i and X_j represent coded values of the independent variables. Design-Expert software statistical package (version 7.0 trial versions for windows, Stat-Ease, Inc. Minneapolis, USA, 2009), was employed for the experimental design to analyse the model and to generate 3D response curves from experimental data. Analysis of variance was employed for the determination of possible interactions of process variables at 95% confidence level. Models were considered suitable when it is "significant" based on analysis of variance (ANOVA), when the lack –of-fit was insignificant with R^2 value above 0.75. The selected models were thereafter optimized on the basis of the optimization criteria: - pressure -within range, temperature- minimum and dynamic time- minimum. The criteria for recovery of responses (YE, TPC, EGCG, and TC yields) were set at maximum.

2.8 High performance liquid chromatography (HPLC)

The method of (Pranathi, Borba, & Jeffrey, 2011) was used for HPLC analysis of catechin from the extracts obtained. The chromatographic conditions of HPLC methods used included the use of almost exclusively, a reversed-phase C18 column; UV-Vis diode array detector, and a binary solvent system containing acidified water (0.1% orthophosphoric acid -solvent A) and a polar organic solvent (acetonitrile -solvent B). Optimized gradient elution order was programmed as follows: 0 min 96%A:4%B; 12 min85%A:15%B; 22 min 75%A:25%B;24 min85%A:15%B; 30 min85%A:15%B and 35. Min 96% A: 4% B. The flow rate and injection volume were set at 1mL/min and 10µL respectively with a post run of 5 minutes. The column temperature was 35^{o}C while the wave length was 280nm using UV detectors (Pranathi et al., 2011).

3.0 RESULTS AND DISCUSSION

3.1 Solvent extraction

In this study the effects of optimized extraction variables namely, pressure temperature and dynamic time on the extraction yield (Y), total phenolic compound (TPC), epigallocatechin (EGCG) and total catechin (TC) of green tea leaves from Cameron highlands Malaysia was investigated with a view to attaining maximum recovery. Two other independent variables, co- solvent and carbon dioxide (CO_2) flow rates were kept constant. The lower limits and upper limits of experimental variables was based on outcome of our preliminary studies which support that for maximum recovery of catechins the pressure should be within the range 10 - 30Mpa with lower temperature limit near that of critical temperature of CO_2 (31.1^{0}C). The dynamic extraction time of 40 -120 minutes was adopted although it shows little effects on the yield of catechins, but that could reduce undue exposure of the catechins to degradation conditions because of the dual effects of temperature on the active ingredients during extraction (Bimakr et al., 2011; Cossuta et al., 2008; Liza et al., 2010; Roop, Dexter, & Irvin, 1989). Table.1. shows fitting of experimental data and the observed values of responses with various combination of independent variable, pressure (x_1), temperature (x_2) and dynamic time (x_3) used for the evaluation of coefficient of second –order polynomial equation while table 2 is the summary of the Fitted Model equation for the yield, TPC, EGCG and TC of the supercritical fluid extraction of green tea leave developed from the coded and actual values

3.1.1 Extraction yields

Tea polyphenolic compounds are known to possess high antioxidant activity. Three different solvents were used to determine the extraction yield, total phenolic content, antioxidant scavenging activity and total catechin of green tea leaves from Cameron highlands, Malaysia. The results are as presented in **Figs.1, 2 &3.** **Fig.1** shows crude extract yield was found to vary from 2.05±0.21 -3.57±0.13%. There was no significant ($p \leq 0.05$) difference between methanol 70% and ethanol 50%. Water produced the list. Total phenolic content was determined by the Folin –Ciocalteu assay. The total phenolic content was in the range of 11.43±0.25 – 22.96g/ 100g (GAE) [**Fig.2].** Ethanol 50% (v/v) produce the highest total phenolic content of 22.96 (g/100g) GAE, whereas methanol (100%) produced the lowest total phenolic content. Total phenolic contents has been previous reported in different types of tea (Hara, et al.; 1995, Chan, Lim, & Chew, 2007). Chan, Lim and Chew reported TPC of between 11.10±0.69 – 19.13±0.13 (g/100g GAE) in Malaysian green and Australian black tea. A comprehensive review on the progress phenolic constituent analysis of tea over a period of 20 years also reported similar results.(Stodt & Engelhardt, 2013). The minor differences observed in this reported may be attributed to analytical methods and climatic factors (Stodt & Engelhardt, 2013).

Fig.3 showed the result of the DDPH assay of the solvent extracts. Methanol70% (v/v) shows the highest antioxidant activity followed by ethanol and methanol 50%. No significant difference was observed between ethanol and methanol 100%. There was high correlation between the total phenolic content and antioxidant capacity test. Interesting this result shows that green tea leaves from Malaysia contains high antioxidants when comparable with other reports on green tea leaves (Chan et al., 2007; Taheri, Sariri, Giahi, & Ghafoori, 2011). The result of this study showed that the type of solvent employed significantly ($p \leq 0.05$) affects the antioxidant potential of green tea extracts.

3.5 Total catechins

The chromatograms obtained from the HPLC analysis of the green tea leaves extracts is presented in fig.6a&b. The chromatogram shows the various catechin monomers identified by comparing the relative retention times from the samples with those of the standards which were made solely from the major catechins namely: Catechin hydrate(C), Epigallocatechin (EGC), epicatechin (EC), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), and epicatechin gallate (ECG).

3.6 The Optimum conditions for the extraction of green tea leaves

Using the design expert software the optimum extraction condition for the four independent variables (extractable crude yield, TPC, EGCG and TC) were established at a pressure (30Mp, temperature(47.99^0C) and dynamic time(40 minutes) with a co- solvent (50% water ethanol) and CO_2 flow rates of 1.5 and 1.0ml/ minutes respectively **Fig.9.** This resulted in total extract yield of 6.65%, TPC of 28.07g/100g, epigallocatechin gallate 263.06mg/g and total catechin 844.25mg/g.

3.7 single factor analysis

In this study, optimization of the major extraction variables (pressure, temperature, and dynamic time) on the extractable yields was carried out. Design Expert software polynomial regression aligned with the results of the experiment. Data from the experimental yields were fitted on to second order-polynomial. The regression equation coefficients and p-values for the extraction of green tea are as presented in **Table3.3a-b** Based on the mathematical models the significance of the factors under investigation and their interactions were examined. It can be observed that the measure of fitness of the data which is represented by; the model fitness ($p < 0.05$), the r^2 values, were above 0.75 (indicating a good fit) and the "lack of fit" value which also measure the fitness of the model for the Y_E, TPC and EGCG were not significant except that of TC.

For the recovery of Y_E , TPC, EGCG and TC three linear coefficients(x_1, x_2, x_3), cross –product effects (x_1x_2 ,x_2x_3) and quadratic effects (x_1^2, x^2_2, x_3^2 $x_1x^2_2$) were all examined ($p < 0.05$) in respect to the response variables. The coefficient of the independent variables that were positive implied that when the independent variables (pressure, temperature and dynamic time) increased, the response variables also increased.

3.3.5 Interactive effects of extraction variable on yields

3.3.5.1 Interactive effects of pressure and temperature and dynamic time on crude yield

The interactive effects of pressure, temperature and dynamic time on green tea leaves in respect to the crude yield, TPC, TC and EGCG are presented in **Figs. 3.8a &b**, **3.9a&b** and **3.10a, b &c**.

The interactive effect of temperature and pressure on crude extract yield is represented in **Fig3.8a.** When the pressure was at 10Mpa, increase in temperature from 40 – 60^oC caused a decrease in crude extract yield slightly (7.59 -7.13%) whereas as the pressure increased to 30Mpa at the same temperature, crude extract yield increased by 2.35%. That is, increase in pressure and at higher temperature results in higher quantitative recoveries of yield as a result of better interaction between the matrix and SC-CO_2 fluid. The interactive effect of dynamic time and pressure on the material ratio of crude extracts is shown in **Fig3.8b**. It was observed that as the pressure increased from 10 – 30Mpa with increase in dynamic time (40 – 120 minutes) the crude extract yield was greatly increased ($p < 0.05$) from 3.08 – 9.14%, showing that increased in dynamic time offers enough time for fresh material to flow within the system giving rise to higher yield. The crude extract yield reached a maximum of 8.40% from the lower point. This means that that interactive effect of pressure and dynamic time had a positive effect on the crude extract yield of the green tea leaves. The increase in extractable yields at a higher pressure may be attributed to increase in green tea leaves solubility in SC-CO_2. Fluid density increases when pressure increased which alter solubility. Increased SC-CO_2 density will result in increase in its dissolving ability. On the other hand when the temperature increased fluid density decreases with resultant increase in solute vapour pressure. Similar report has been made in previous researches (Gomes, Mata, & Rodrigues, 2007; Sonsuzer, Sahin, & Yilmaz, 2004; Zarena, Sachindra, & Udaya Sankar, 2012). The influence of temperature is based on the fact that temperature increase the analytes vapour pressure which leads to low yield of extractable compounds (E. Reverchon, 2006; Roberto et al., 2010), when pressure is at the critical range, the effect of temperature on solute solubility differs. Near the system critical pressure the fluid density is very sensitive to temperature therefore a moderate increase in temperature can lead to large decrease in fluid density with consequent reduction in solubility (Roop, Akgerman, Dexter, 1989a). On the other hand, increase in temperature can accelerate mass transfer leading to improved extraction yield, whereas increasing the vapour pressure increase the tendency to extract the compounds as they pass

through the supercritical phase. This may be the reason for the positive roles played by temperature on the extractable yield in this study. We observed as the dynamic time increased there was a steady increase in the extractable yields of crude yield and EGCG. However, in the case of TPC and TC we found that dynamic time lead to increase only to a certain point thereafter it played no positive role. However, it is expected that dynamic time should be kept as minimum as possible for economic reasons. This is because SFE process involves reasonable cost of investment and the extraction time may affect the total extraction cost. The use of minimum static time near the highest rate of recovery is economically viable for functional food manufacturer, the choice of minimum static time and dynamic time in our study.

3.3.5.2 Interactive effects of dynamic time and pressure and temperature on phenolic content (TPC) yield

Fig.3.9a described the response surface interactive effects of pressure and temperature on total phenolic contents (TPC). It was observed that at pressure of 10Mpa, when temperature increased from 40 – 60^{o}C, there was reduction in TPC yield from 23.33 - 22,82g/ 100gGAE (2.23%), but as the pressure increased to 30Mpa at the same temperature condition, there was a slight increase (2.02%) TPC yield. This is because when there is higher pressure and temperature there is a higher interaction in the fluid density and the matrix which was responsible for quantitative recovery of TPC. Similar trend has been reported (Bimakr et al., 2011; Roop, Akgerman, Dexter, 1989b). The interactive effect of temperature and dynamic time on TPC is shown in **Fig3.9b**. At a lower dynamic time of 40 minutes the recovery of TPC was lower as the temperature increased from 40 – 60^{o}C.Whereas when the dynamic time increased to 120 minutes the yield of the TPC increased by 6.52% (24.40 – 26.06g/100gGAE). Increasing the dynamic time permits solvent to flow over the fix bed of powder until mass transfer is effective. This has been corroborated in similar studies by previous researchers (Arias et al., 2009; Bimakr et al., 2011; Le Floch, Tena, A. Ríos, 1998).

However, it is always difficult to predict the effects of temperature on extraction yield because high temperature can decrease salivation power couple with the fact that phenolics compounds are heat sensitive. Le Floch, Tena, A. Ríos,(1998); Bimakr, Rahman, Taip, Chuan, Ganjloo & Selamat, (2009) had reported similar findings on the effects of temperature on phenolic extracts, whereas Leal et al., (2007) and S. Scalia, L. Giuffreda, (1999) reported that temperature did not influence extraction yields. The negative coefficient value of temperature observed **(Table2)** in this study agrees with their findings. This shows that temperature can play dual roles in SC-CO2 extraction. Therefore temperature becomes an important factor to be control for maximum yield of phenolic compounds during extraction. The influence of dynamic time on extraction of phenolic compounds have been reported (Bimakr et al., 2011; Le Floch, Tena, Ríos, 1998; Bimakr, Rahman, Taip, Chuan, Ganjloo & Selamat, 2009). Increasing dynamic time increase yield because fresh solvent passes over the fix bed of green tea powder until effective mass transfer between green tea powder and SC-CO_2 is attained. In this study we found that dynamic time affected the phenolic yield significantly ($p \geq 0.05$).

3.3.5.3 Interactive effects of pressure, temperature and dynamic time on epigallocatechin gallate (EGCG)

The interactive effect of pressure, temperature and time on EGCG yield is shown in **Figs.3.10a-b. Fig.3.10a** represents the interactive effect of pressure and temperature on EGCG yield. At a pressure 10Mpa, recovery yield of EGCG increased slightly by 1.87% as the temperature increase from 40 -60^{o}C. When the pressure increased to 30Mpa with increased temperature there was increase in EGCG yield from 1.87 -3.08%. Thus higher pressure along with increased temperature give rise quantitative recoveries and better interaction between the matrix and the fluid. The interactive effect of dynamic time and pressure is presented in **Fig.3.10b**. There was no significant effect on the recovery of EGCG ($p < 0.05$) at the initial stage, but as the dynamic time decreased with increased pressure the effect become positive with 4.07% increase in EGCG from 0.34%. This can be seen as indicated by a positive value of the coefficient of 2.0 **(Table3.3b).** Fresh solvent passing through the green tea fixed beds as a result of which it gives rise to higher recovery of EGCG. as explained earlier. There was no suitable model established for the determination of the total catechin yield of the green tea leaves extracts. This may be as a result of non-stable nature the and wide variation in the composition of the five major catechin components that form the total catechin.

In this study we observed that pressure was the most significant factor in the recovery of all the response variables in SFE. When the pressure increased from 10Mpa - 30Mpa, there was significant ($p< 0.05$) increase in the value of Y_E, TPC, EGCG and TC from 3.17% - 8.76%, 21.96 - 27.26g/100g GAE. 218.96 -264.50mg/ 100ml and 557.06 - 818.09 mg/ 100ml respectively. The increase in extractable yields at a higher pressure is attributed to increase in green tea leaves solubility in SC-CO_2. Fluid density increases when pressure increased which in turn alters solubility of the green tea leaves. Increased SC-CO_2 density will result in increase in its dissolving ability. On the other hand when there is increase in temperature fluid density decreases with resultant increase in solute vapour pressure. Similar report have been made by other researchers during extraction of phenolic compounds (Zarena, Manohar, & Udaya Sankar, 2010; Zarena et al., 2012). The solubility of the solute may remain constant depending on whether the solvent density or solute vapour pressure predominate as a factor. At higher pressure the solute volatility and diffusivity increase as the temperature rises, whereas the SC-CO_2 does not depend so markedly on the temperature, therefore increase in vapour pressure of the solute prevails and the solubility rises. I also found that an increase in temperature when the density of CO_2 is kept constant pressure decreases, as the temperature increase the

solubility power for SC-CO_2 decreases. The influence of temperature is based on the fact that temperature increase the analytes vapour pressure and that leads to the low yield of the extractable compound. It has been reported that when temperature increases marginally it may result in large reduction of the fluid density with a resultant reduction in solute solubility (Reverchon, 2006; Ozkal, Roberto *et al.*, 2010; Song *et al.*, 2010). When the pressure is at the critical range, the effect of temperature on solute solubility differs. Near the system critical pressure the fluid density is very sensitive to temperature therefore a moderate increase in temperature can lead to a large decrease in fluid density with consequent reduction in the solubility (Roop *et al*., 1989). On the other hand increase in temperature can accelerate mass transfer thereby leading to improved extraction yield; while increasing the vapour pressure of the extractable compounds, the tendency of the compound to be extracted will increase as they pass through the supercritical phase. This may be the reason for the positive roles played by temperature on the extractable yields in this study. It is always difficult to predict the effects temperature on the extraction yield of polyphenolic compounds because high temperature can decrease salvation power couple with the fact that phenolics are heat sensitive. This study agrees with(Bimakr et al., 2011). However, when extracting sweet basil, Leal et al.,(2007), reported that temperature did not influence extraction of compounds. The negative coefficient value of temperature **(Table3.2)** agrees with their findings. The dual roles temperature played in this study support the assertions that temperature is an important factor in SC- CO_2 which must be control for maximum yields of phenolic compounds.

In the overall, pressure and temperature played a significant role in the extraction of green tea catechins.

3.3.6. The Optimum conditions for the extraction of green tea leaves

The optimum extraction conditions were obtained using the design expert software considering quantitative yields of the four independent variables (extractable crude yield, TPC, EGCG and TC). Optimum extraction conditions were established at pressure (30Mp, temperature (47.99^{o}C) and dynamic time (40 minutes) with a co- solvent of 50% (v/v)), CO_2 flow rates of 1.5mL and 1.0mL per minutes respectively. While deciding the minimum and optimum values of the response variables maximum values were selected because our aim is to obtain maximum recovery. Optimum extraction were reported as: crude extract yields - 6.65%, TPC - 28.07g/ (100gGAE), epigallocatechin gallate (EGCG) -263.06mg/g and total catechin (TC) - 844.25mg/g **(Fig3.11)**

3.3.7 Validation of the optimum extraction condition model

The response values obtained using the models were evaluated by conducting 5 independent experiments using the set of conditions: pressure of 30Mpa, temperature 48^{o}C, time of 40 minutes, CO_2, and co-solvent flow rate of 1.5mL and 1mL per minute. The results obtained were analysed using analysis of variance and was compared with the optimum response values obtained from the design Expert software **(Table3.6).** The result shows that the model was good for the extraction technique that was used.

3.3.8 Comparison between SFE extracts and the catechin polyphenols obtained by conventional solvent extraction

The extractable yields from the supercritical fluid conventional solvent extraction from the green tea leaves were compared and the results were as presented in **Table3.6.** The SFE technique gave higher extractable yields compared to the conventional solvent method. Similar result has been reported by Gadkari et al., (2013). The mean yield obtained by SFE was much higher than the yield obtained by solvent extraction as shown. It was also observed that the total phenolic compounds recovered from SFE were higher than those of solvent extraction **(Table3.6).** Extracted values of total catechin was within range of 234.50 -887.73mg/100ml; while that of solvent extracts was between 143.64- 291.20mg/100ml.

Table 3.6 comparison between SFE and conventional solvent extracts from green tea leaves powder showing recovery yields.

CONCLUSION

Extraction of catechin was carried out from green tea leaves from Cameron highlands Malaysia using supercritical fluid carbon dioxide (SC-CO_2) using ethanol co-solvent (50% v/v) at optimized conditions of pressure, temperature and dynamic time. Extraction yield of crude extract (Y_E), total phenolic compounds (TPC), epigallocatechin gallate (EGCGC), and total catechin (TC) were influenced by increased pressure, temperature and dynamic time with pressure and temperature exerting the greatest influence. RSM was successfully used for optimizing the parameters in SFE. The optimum extraction condition for response variables from green tea leave were 30Mpa, 48^{o}C, and 40minutes with extraction yields (Y_E) of 6.65% TPC of 28.07g/(100gGAE), EGCG of 30.11mg/g and total catechin (TC) of 99.07±15.10mg/g. The results obtained from supercritical fluid extraction was compared with that of the conventional organic solvents. The catechin phenolic compounds obtained from organic solvent and water were in the range of; 2.05 -3.57%, TPC was in the range of 11.43 – 22.96g/100gGAE, EGCG ranged 22.55 – 38.55mg/g, and TC59.75 -79.33mg/g. The results from conventional organic solvents were lower than those obtained by supercritical fluid extraction. This shows that the SFE extraction technique employed was better for extraction of catechin from green tea. The most significant factor in the extraction process of SFE was Pressure followed by temperature. The result also revealed that green tea from Cameron highlands Malaysia can favourably be compared with tea obtained from other part of the world in terms of total catechins contents and antioxidant activity and can be used as functional food or pharmaceutical ingredients.

ACKNOWLEDGEMENT

The authors will like to thank The University Putra Malaysia for the financial support to the faculty of Food Science and Technology. We are so grateful to Associate prof. Dr. Badlishah S.B., who secured the grant for the research (FRGS No. 5524560).

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Table I
Experimental point of the design expert and the experimental data

SD	RUN	A:pressure (x_1X_1)	B:Temperature (x_2X_2)	C: Dynamic time. ($_xX_3$)	Yield (%)	TPC g/100g GAE	Total catechins mg/100ml	EGCG mg/100 ml
20	1	(0)20.00	(0)50.00	(0)80.00	6.45	25.58	732.95	243.53
19	2	(0)20.00	(0)50.00	(0) 80.00	6.95	26.5	733.25	242.52
17	3	(0)20.00	(0)50.00	(0) 80.00	6.51	25.6	733.75	240.51
15	4	(0)20.00	(0)50.00	(0) 80.00	6.56	23.55	733.55	242.52
18	5	(0)20.00	(0)50.00	(0) 80.00	6.45	25.55	733.44	255.31
16	6	(0)20.00	(0)50.00	(0) 80.00	6.54	25.55	733.74	242.55
9	7	(-1.68)3.18	(0)50.00	(0) 80.00	0.15	18.75	234.5	212.11
14	8	(0)20.00	(0)50.00	(1.68)147.27	6.95	26.71	729.55	239.49
12	9	(0)20.00	(1.68)66.62	(0)80.00	9.95	23.41	721.552	229.52
13	10	(0)20.00	(0)50.00	(-1.682)12.73	1.33	28.57	720.55	229.55
11	11	(0.00)20.00	(-1.682)33.18	(0)80.00	5.45	22.33	732.75	240.51
10	12	(1.68)36.82	(0.00)50.00	(0)80.00	9.56	27.65	869.94	284.85
5	13	(-1)10.00	(-1)40.00	(1)120.00	9.75	23.25	726.5	213.4
7	14	(-1)10.00	(1)60.00	(1)120.00	9.25	24.14	642.31	225.5
3	15	(-1)10.00	(1)60.00	(-1)40.00	3.35	22.55	649.75	226.5
1	16	(-1)10.00	(-1)40.00	(-1)40.00	3.7	25.4	668.15	212.5
2	17	(1)30.00	(-1)40.00	(-1)40.00	1.04	25.02	875.85	257.5
4	18	(1)30.00	(1)60.00	(-1)40.00	6.75	23.59	867.53	274.71
6	19	(1)30.00	(-1)40.00	(1)120.00	1.92	25.12	755.68	265.55
8	20	(1)30.00	(1)60.00	(1)120.00	8.05	26.64	887.73	282.2

Table 2a.

Analysis of variance of fitted models for crude yield and TPC

Variables	Y_E			TPC		
	Coefficient	F	Prob<F	Coefficient	F	Prob<F
X_1	2.80	1676.48	0.0001*	2.65	39.60	0.0001*
X_2	1.36	954.92	0.0001*	-3.93	2.11	ns
X_3	1.73	1541.87	0.0001*	-0.04	0.02	ns
X_1X_2	1.58	762.22	0.0001*	0.26	0.52	ns
X_1X_3	-1.22	451.80	0.0001*	-	-	ns
X_1^2	-0.61	206.05	0.00018*	0.79	8.79	0.0142*
X_2^2	0.39	83.59	0.0001*	-0.90	11.75	0.0067*
X_3^2	-0.87	410.45	0.0001*	0.78	8.75	0.0143*
$X_1X_2^2$	-3.83	1844.32	0.0001*	-2.02	13.48	0.045*

Pressure(X_1), temperature(X_2), dynamic time(X_3), *--Significant ($p<0.05$), ns-- not significant, R2-- 0.9984, pred.R^2 – (0.888-0.9951), lack of fit- not significant

Table 2b
Analysis of variance of fitted models for EGCG and TC

Variables	EGCG			Total catechin(TC)		
	Coefficient	F	Prob<F	Coefficien t	F	Prob<F
X_1	23.78	112.59	0.0001*	129.50	33.39	0.0002*
X_2	3.06	1.87	ns	0.17	5.684E-05	ns
X_3	2.38	0.13	ns	-2.48	0.012	Ns
X_1X_2	1.01	0.12	ns			
X_1X_3	2.00	0.45	ns			
X_2X_3	-0.27	8.34E-003	ns			
X_1^2	2.62	1.43	ns			
X_2^2	-2.14	0.96	ns			
X_3^2	-2.31	1.12	ns			

Pressure(X_1), temperature(X_2), dynamic time(X_3), *--Significant ($p<0.05$), ns-- not significant, R2 - (7652- 0.9984), pred.R^2 – (0.6797-0.9951), lack of fit- not significant

Table III
Result of validation of fitness of the RSM model.

Extract	Theoretical value	Experimental value
Y (%)	6.65	5.75±1.25
TPC(g/100gGAE)	28.07	27.32±1.75
EGCG(mg/100ml)	263.06	245.45±18.76
TC(mg/100ml)	844.25	865.25±285.75

Table IV
Comparison between SFE and conventional solvent extracts from green tea leaves powder showing recovery yields from Cameron highlands malaysia

	Extractable Yields			
Method/solvent (%)	Y (%)	TPC (g/100gGAE)	EGCG (mg/g)	TC (mg/g)
SFE	6.65±0.20[a]	28.07±0.25[a]	44.11±5.35[a]	96.67±15.10a
ethanol 100	2.48±0.30[c]	11.97±0.21[c]	28.75±0.14[f]	59.75±0.60[g]
ethanol 70	2.32±0.32[cd]	16.31±0.18[d]	34.45±0.49[e]	70.22±0.28[e]
ethanol 50	3.48±0.12[b]	22.96±0.30[b]	37.25±0.48[c]	77.85±0.24[c]
methanol 100	2.36±0.30[bc]	11.43±0.15[c]	22.55±0.38[g]	45.22±0.32[h]
methanol 70	3.57±0.13[b]	21.23±0.17[b]	38.55±1.17[b]	79.33±0.37[b]
methanol 50	2.23±0.32[d]	16.60±0.32e	36.75±0.17[c]	68.75±0.50[f]
water	2.05±0.021[e]	15.55±0.25[g]	35.201±0.48[d]	75.05±0.50[d]

Means that do not share a letter along column are significantly different All value are mean of three independent determinations ±SD. **Y**–crude extract yield, **TPC** –Total phenolic content, **EC** –epicatechin, **EGC**-epigallocatechin, **EGCG** –epigallocatechin gallate, **GCG** –gallocatechin gallate, **TC**- total catechin.

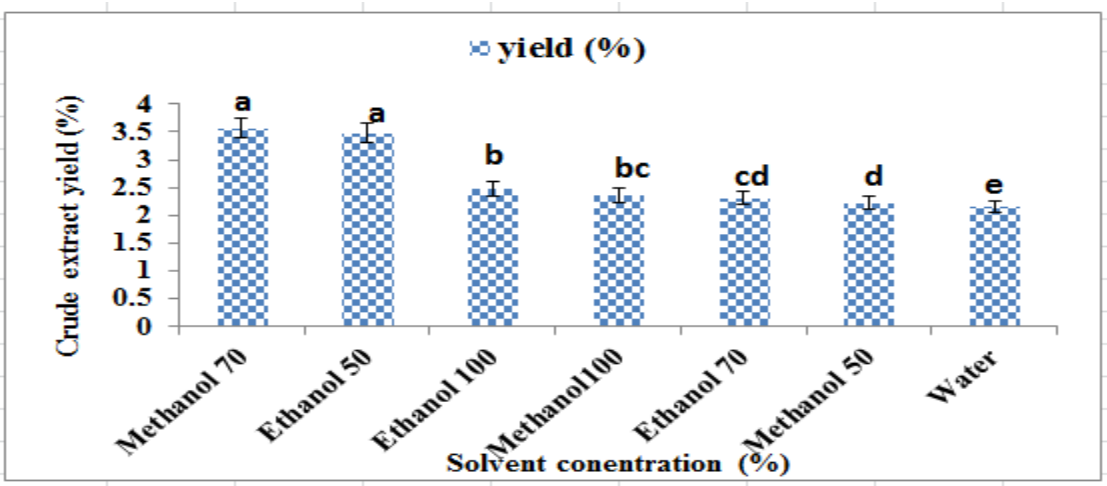


Fig. 1. crude extract yield from green tea (*Camellia sinensis*) using different organic solvent Means that do not share a letter are significantly ($p \geq 0.05$) different. All values are mean of three independent determinations.

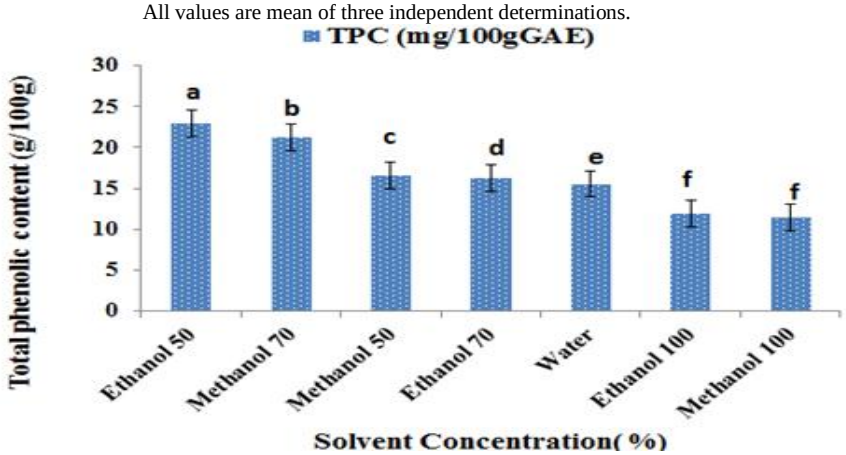


Fig. 2. Total phenolic content of solvent extraction from green tea leaves powder. Means that do not share a letter are significantly ($p \geq 0.05$) different. All values are mean of three independent determinations.

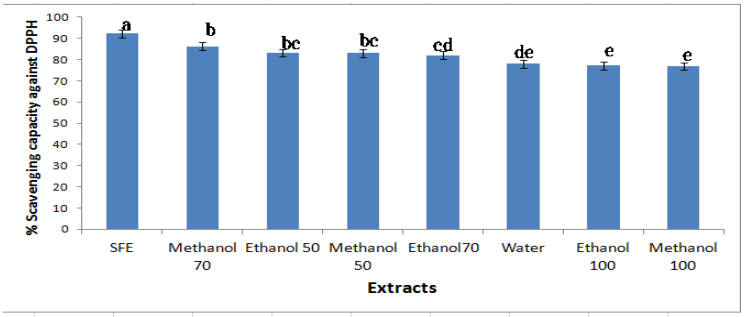


Fig. 3. Antioxidant power of solvent extracts of green tea catechin as measured by DPPH assay. Values are means (± SD) of replicate measurement for two individual determinations (n =

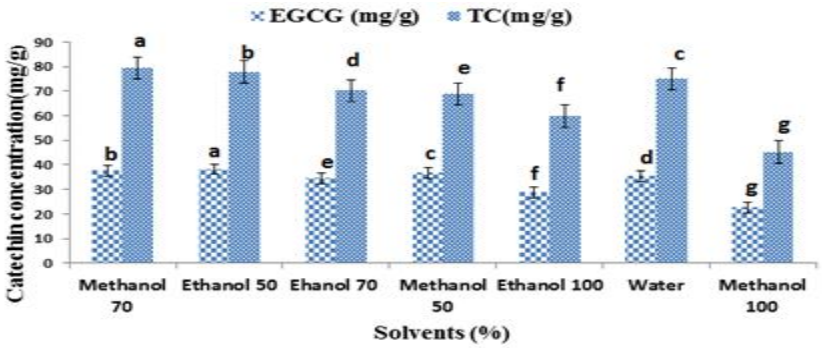
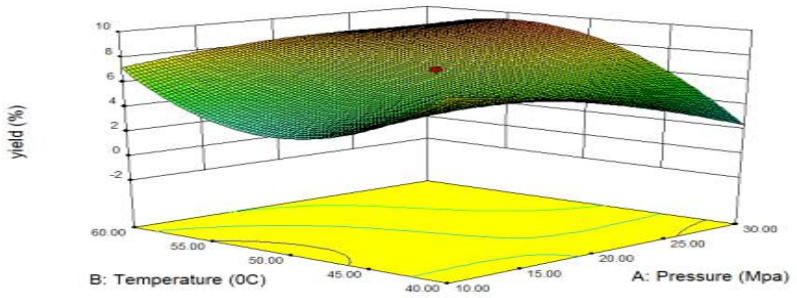


Fig. 4. effect of solvent on total catechin (TC) and epigallocatechin gallate (EGCG) assay. Values are means of replicate measurement for two individual determinations (n =



(b) Fig. 5a 3D response surface plot showing the interaction effect of pressure(x_1) and dynamic time(x_3) at a fixed level of temperature on crude Yield

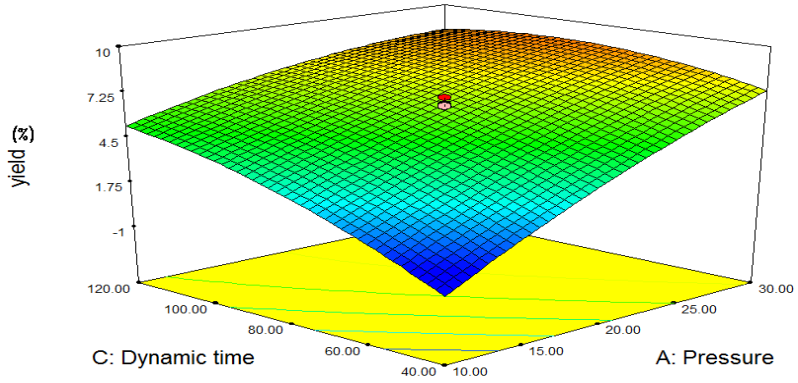


Fig. 5b .3D- response surface plot showing the interaction of pressure (x_1) and dynamic time(x_3) on crude yield of green tea leaves,

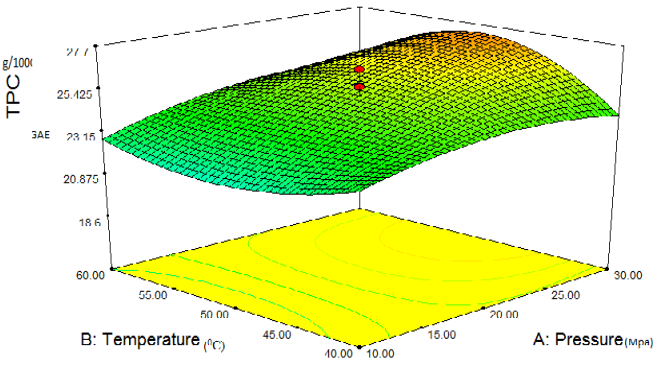


Fig. 6a. 3D-response surface plot showing the interaction effects pressure (x_1) and temperature(x_2) on TPC of green tea leaves

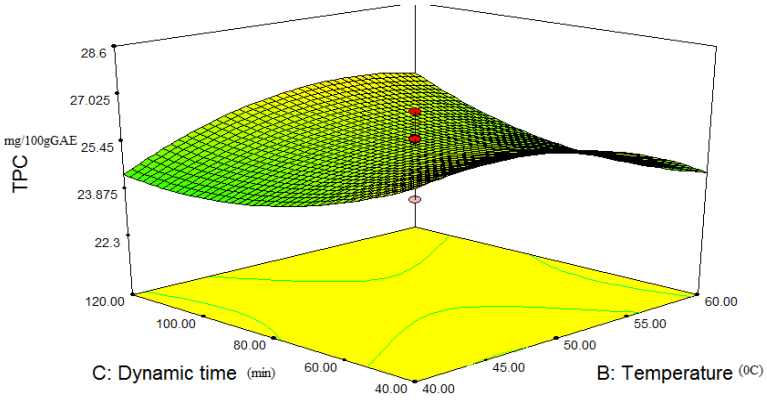


Fig. 6b. 3D-response surface plot showing the interaction effects of temperature (X_2) and dynamic time (X_3) on TPC yield

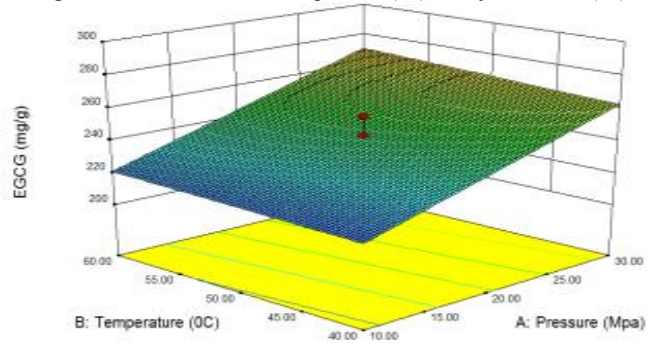


Fig. 7 a. 3D-response surface plot showing the interaction effects of pressure (x_1) and temperature (x_2) on EGCG of green tea leaves

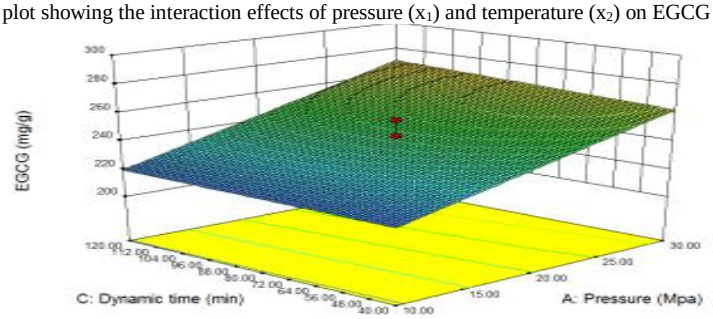


Fig. 7b. 3D-response surface plot showing the interaction effects of pressure (x_1) and dynamic time (x_3) on EGCG of green tea leaves,

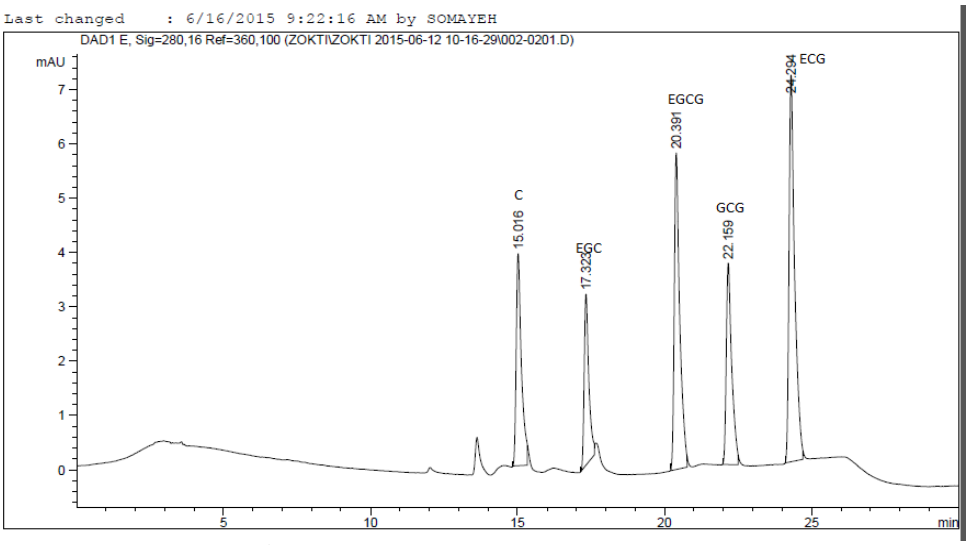


Fig. 8 a. Chromatograms representing the composition of catechins standards using HPLC analysis. **C**-catechin**; EGC**-epigallocatechin; **EC**- epicatechin; **EGCG**- epigallocatechin gallate; **GCG**- gallacatechin gallate; **ECG**-epicatechin gallate

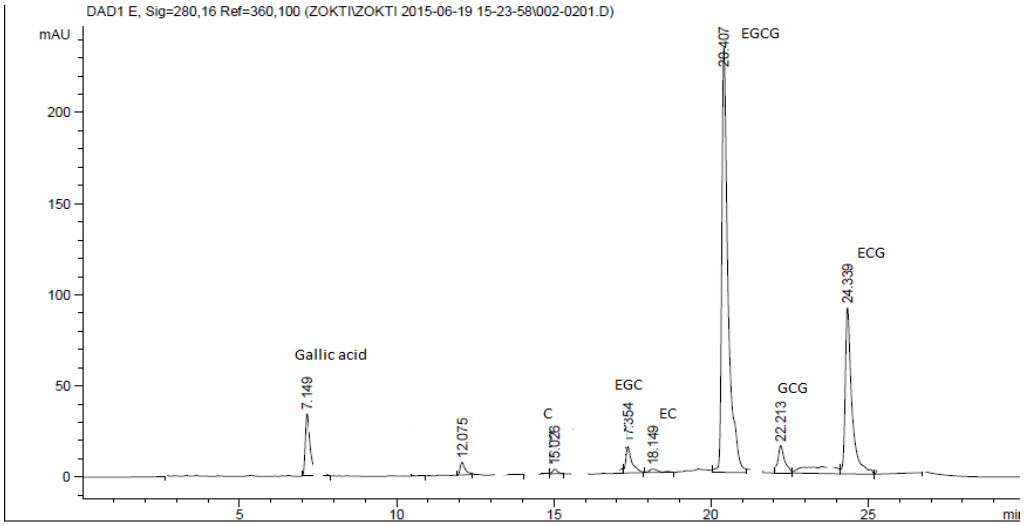


Fig. 8b. Chromatograms representing components of catechin monomers obtained from SFE and solvent extraction during **HPLC** Analysis ; **C**-catechin**; EGC**-epigallocatechin; **EC**- epicatechin; **EGCG**- epigallocatechin gallate; **GCG**- gallacatechin gallate; **ECG**-epicatechin gallate

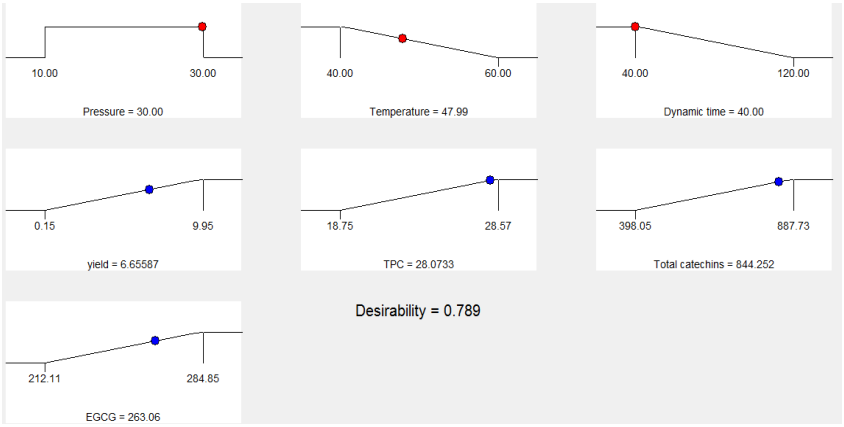


Fig. 9. Optimumized conditions of independent and the response variables derived from design expert software.