BEE-BASED SENSOR USING *APIS MELLIFERA* FOR DETECTION OF *ANDROGRAPHIS PANICULATA* VOLATILE COMPOUNDS

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ABSTRACT

Honey bees can be trained to be sniffer bees due to the presence of high number of odorant receptors (170 odorant receptors) in their heads. This unique characteristic enables them to detect scent down to part per trillion level. In this study, localized honey bees (*Apis mellifera*) were trained by using the method of classical Pavlovian conditioning. The proboscis extension reflex (PER) of sniffer bees towards the target odor was observed and recorded. The phytochemical profile of *Andrographis paniculata* (*A. paniculata*) was constructed using headspace solid-phase microextraction coupled with gas chromatography integrated with mass spectrometer method. The volatile marker compounds were identified. The sniffing capacity of the sniffer bees was determined by varying the heating temperature from 50-120 °C, the weight of plant material from 20-100 mg and the percentage (20-100%) of the target herbal sample in the mixture of *A. paniculata* and *Clinacanthus nutans* (*C. nutans*). *C. nutans* is an herbal plant which is morphologically similar to *A. paniculata* and it also belongs to the Acanthaceae family. The efficiency, accuracy and sensitivity of sniffer bees were analyzed and validated statistically. The success rate of sniffer bees for heating temperatures was approximately 90 %. The success rate for minimum weight of plant sample, 20 mg was 50 %. The success rate percentage of target herbal sample increased when the percentage of *A. paniculata* was proportionally increased. Compounds such as caryophyllene, β-elemene, 3,3-dimethylhexane, apiol, 6,10,14-trimethyl-2-pentadecanone and dihydroactinidiolide were detected in the gaseous mixture. The kinetics of volatile marker compounds released from the plant samples were studied to predict the concentrations of the volatile marker compounds for sniffer bee detection at 85 °C. Second-order and two-site kinetic models were selected because of the kinetic data of these volatile marker compounds fitted well to these models ($R^2 > 0.9$).

ABSTRAK

Lebah madu boleh dilatih menjadi lebah pengesan kerana kehadiran reseptor bau yang banyak (170 reseptor bau) dalam kepala mereka. Ciri unik ini membolehkan mereka mengesan bau sampai ke tahap part per trillion. Dalam kajian ini, lebah madu (*Apis mellifera*) telah dilatih dengan kaedah classical Pavlovian conditioning. Tindakbalas pengeluaran lidah (PER) lebah pengesan terhadap bau sasaran telah diperhatikan dan dicatatkan. Profil fitokimia hempedu bumi *(A. paniculata)* telah dibangunkan melalui kaedah pengekstrakan mikro fasa pepejal ruang tutupan yang digandingkan dengan kromatografi gas dan spektrometer jisim. Sebatian-sebatian penanda yang mudah meruap telah dikenalpasti. Keupayaan lebah pengesan untuk mengesan bau telah ditentukan dengan mengubah suhu pemanasan dari 50 ℃ ke 120 ℃, jisim bahan tumbuhan dari 20-100 mg dan peratusan (20-100 %) sampel herba sasaran dalam campuran hempedu bumi (*A. paniculata*) dan belalai gajah (*C. nutans*). Belalai gajah merupakan tumbuhan herba yang mempunyai kesamaan dengan hempedu bumi dari segi morfologi dan ia juga digolongkan kepada keluarga Acanthaceae. Kecekapan, ketepatan dan kepekaan lebah pengesan telah dianalisa dan disahkan secara statistik. Kadar kejayaan untuk pelbagai suhu pemanasan adalah sekitar 90 %. Kadar kejayaan bagi jisim minimum tumbuhan 20 mg adalah sekitar 50 %. Peratusan kadar kejayaan untuk mengesan sampel herba sasaran meningkat apabila peratusan hempedu bumi dalam campuran meningkat secara berkadar. Cariofillene, β-elemene, 3,3-dimetilheksana, apiol, 6,10,14-trimetil-2-pentadekanona dan dihidroaktinidiolida adalah sebatian yang dikesan dalam campuran gas. Kinetik sebatian-sebatian penanda mudah meruap yang dikeluarkan daripada sampel tumbuhan telah dikaji untuk meramal kepekatan sebatian-sebatian penanda yang mudah meruap bagi pengesanan lebah pengesan pada 85 ℃. Model kinetic tertib kedua dan model dwi tapak telah dipilih kerana data kinetik dari sebatian-sebatian penanda yang mudah meruap bersesuaian dengan model-model tersebut (R^2 > 0.9).

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LIST OF SYMBOLS

n	-	Number of honey bees positively respond to target odorant
N	-	Total number of honey bees in a training
N_a	-	Number of sniffer bees correctly recognize target odorant
N_A	-	Total number of sniffer bees in a training
N_t	-	Number of sniffer bees positively respond to target odorant
N_T	-	Total number of sniffer bees in a training

LIST OF ABBREVIATIONS

AL	-	Antennal lobe
CR	-	Conditioned response
CS	-	Conditioned stimulus
eLTM	-	Early long-term memory
GC	-	Gas chromatography
HPLC	-	High performance liquid chromatography
HS	-	Headspace
KCs	-	Kenyon cells
LH	-	Lateral horn
lLTM	-	Late long-term memory
LNs	-	Local interneurons
LTM	-	Long-term memory
MBs	-	Mushroom bodies
MS	-	Mass spectrometer
MTM	-	Mid-term memory
OBPs	-	Odorant-binding proteins
ORs	-	Olfactory receptors
ORCs	-	Olfactory receptor cells
ORNs	-	Olfactory receptor neurons
OSNs	-	Olfactory sensory neurons
PBPs	-	Pheromone binding proteins
PER	-	Proboscis Extension Reflex
PNs	-	Projection neurons
R^2	-	Coefficient of determination
RMSE	-	Root mean square error

SPME	-	Solid phase micro-extraction
STM	-	Short-term memory
TB	-	Tuberculosis
TNT	-	Trinitrotoluene
UR	-	Unconditioned response
US	-	Unconditioned stimulus

CHAPTER 1

INTRODUCTION

1.1 Research Background

Honey bee possesses several key criteria to be an excellent sniffer. Honey bees (*Apis mellifera*) are subset of bees in the genus *Apis*. Honey bees have 170 odorant receptors and a pair of mushroom bodies (Robertson and Wanner, 2006, Farris et al., 1999). Therefore, they can differentiate odors better than fruit flies and mosquitoes which only have 62 and 79 odor receptors respectively (Clyne et al., 1999, Vosshall et al., 1999, Hill et al., 2002). Besides their outstanding ability to distinguish smells, their sensitivity to odors is also excellent. Honey bees can be trained to detect scents down to parts per trillion level (Bromenshenk et al., 2003). The mushroom bodies assist honey bees to keep their memory about the scent, and thus they are able to remember the scent which can last for several days and even for their entire life (Menzel, 1999, Schröter and Menzel, 2003). The life spans of honey bees are different; workers live 3-6 weeks during spring and summer, but about 4 months during winter. For bee queen, her lifespan is around 2-3 years, but less than 1 year in commercial hives. There is not much information about the life span of drones (Page and Peng, 2001).

Due to their extraordinary abilities, honey bees can be used to replace sniffer dogs. Defense Advanced Research Projects Agency (DARPA), an agency of United States Department of Defense had funded a project in 2004 by utilizing honey bees for explosives detection. It was found that they could detect trinitrotoluene (TNT) at the level of part per trillion (ppt). Besides that, Inscentinel Ltd., a company which specializes in the development of insect olfaction technologies for the detection of trace chemicals discovered that honey bees were able to detect 2,4-dinitrotoluene (DNT) down to at least 78 ppt. In this case, honey bees are likely to be superior than sniffer dogs. In 2008, the company has invented Vasor136 (biosensor) which can house 36 bees to detect 6 different chemicals simultaneously. According to Suckling and Sagar (2011), honey bees could be trained to detect the scent of *Mycobacterium tuberculosis* through human breath. A person who is suffering from Tuberculosis (TB) can be determined in a faster and more sensitive manner using sniffer bees. In agricultural industry, honey bees were also used to detect the presence of Mediterranean fruit fly (*Ceratitiscapitata Wiedemann*) larvae in Valencia oranges at early stage of maturity (Chamberlain et al., 2012). Behavioral studies have demonstrated that bees are able to recognize learned odors within <200ms (Krofczik et al., 2009). This characteristic makes them manage to response fast and immediately while exposed to target odorant. Therefore, sniffer bees have been getting actively applied in medical, security and agricultural industries for the last few years. This is the first study to report the use of sniffer bees for herbal plant recognition.

In this research, honey bees are trained by classical Pavlovian conditioning method to detect *Andrographis paniculata* (Hempedu Bumi). *A. paniculata* is an erect herb that widely distributed in South East Asia. The whole plant is traditionally used as an anti-inflammatory and antipyretic folk medicine for the treatment of fever, cold, laryngitis, diarrhoea, and inflammation (Sheeja et al., 2006, Suebsasana et al., 2009). The extract of *A.paniculata* and its major *ent*-labdane diterpenoids have been shown to display antiviral (Wiart et al., 2005), bacteriostatic (Mishra et al., 2009), immunostimulatory (Ajaya Kumar et al., 2004), as well as hepatoprotective and hepatostimulating activities (Kapil et al., 1993) in scientific studies.

In the training of honey bees, their antenna will be touched by cotton bud dipped with sucrose solution when the bees are exposed to the odor of thermally treated *A. paniculata*. The bees will show Proboscis Extension Reflex (PER), which is sticking out their tongues and sugar solution is given to them immediately as a reward.

By repeating this procedure for several times, honey bees will associate the odor of *A. paniculata* with sugar reward. They will automatically stick out their tongues whenever they are exposed to the scent of *A. paniculata* even after a couple of days after training. Honey bees which are successfully trained (sniffer bees) can be further studied and developed into bee-sensor for the application in herbal and food industries in the future.

The kinetics of volatile marker compounds was studied to estimate the shortest time for sniffer bees to detect the volatile marker compounds during the heating process of *A. paniculata* at the optimum temperature. This kinetic model is very important to relate the concentration of volatile marker compounds to the Proboscis Extension Reflex (PER) of bees for better performance of sniffer bees in carrying out their sniffing task.

1.2 Research Problem

The identification of *A. paniculata* is usually carried out by solvent extraction and followed by high performance liquid chromatographic analysis. This conventional technique is time-consuming and environmental unfriendly since lot of solvent is required for plant extraction. The detection of *A. paniculata* based on gas phase sample is relatively seldom studied by researchers. This could be the difficulty in gas sample collection and data reproducibility. The plant sample is heated to generate volatile compounds which are then collected for gas chromatography injection. Indeed, this gas phase technique is simpler, cost effective and time-saving if sample lost could be reduced to a minimum level. Nevertheless, this gas chromatography method must be validated before use. *Andrographis paniculata* is becoming popular due to its significant medical value. It is manufactured into pharmaceutical products for medical purpose. Therefore, the purity of raw material becomes the largest concern when comes to the formulation of medical products and a rapid screening of the raw material is a must to prevent adulteration.

Honey bees have been proven by many researchers to be excellent sniffer bees. The performance of sniffer bees is likely to be comparable or better than sniffer dogs (Gazit and Terkel, 2003, Bromenshenk et al., 2003, Angle et al., 2016). It was found that honey bees can be trained to detect explosives, drugs, landmines, infestation of food by insects and disease through the breath of Tuberculosis (TB) patients. However, there is still no report showing its usage and effectiveness in sniffing herbal plants. Therefore, the sensitivity and accuracy of sniffing capacity of sniffer bees to recognize *A. paniculata* is not yet explored and quantified.

1.3 Research Objectives

This research is conducted to determine the sniffing capacity of sniffer bees for *A. paniculata* detection. Honey bees are trained by Pavlovian conditioning method to detect and recognize the volatile marker compounds released from thermally-treated *A. paniculata*. The reliability, sensitivity and persistency of sniffer bees are validated to prove that the utilization of sniffer bees in herbal plant detection is feasible and effective.

There are three objectives for this study. The objectives are:

i. To determine the sniffing capacity of honey bees in terms of reliability, sensitivity and persistency for the detection of volatile marker compounds from thermal-treated *A. paniculata* (Hempedu Bumi).
ii. To construct the volatile phytochemical profile of *A. paniculata*.
iii. To determine the kinetics of the release of key volatile marker compounds from thermally-treated *A. paniculata*.

1.4 Research Scopes

i. To determine the efficiency, accuracy and sensitivity limit of sniffer bees for *A. paniculata* detection by introducing other herb in the same family of Acanthaceae, namely *Clinacanthus nutans* (Sabah snake grass) as sample adulterant.

ii. To validate the persistency characteristic of sniffer bees by keeping the same batch of sniffer bees for 5 days.

iii. To construct the volatile phytochemical profile of *A. paniculata* at heating temperature varied from 50-120°C and detected by gas chromatography integrated with mass spectrometer.

iv. To identify the relationship of volatile marker compounds concentration and heating temperature with the performance of sniffer bees.

v. To determine the kinetics of the release of volatile marker compounds and predict the shortest sample heating time for sufficient amount of volatile marker compounds release at the optimized heating temperature (85°C).

1.5 Research Significance

The prominent significance of the study is the use of honey bees to perform task that is difficult and less accurate to be carried out by mankind. The sniffing capacity of honey bees can be scientifically proven for the detection or differentiation of herbal plant. This non-destructive exploration uses honey bees as biological detector to detect the target odor. The sensitivity capacity of *A. mellifera* could be established for detection of *A. paniculata*.

This research generated fundamental data for the development of bee-based sensor. This sensor is very useful for herbal and/or food industries for quality control purposes. When raw herbal materials are used for research purpose, bee-based sensor would be a great tool to ensure the quality of raw herbal materials can be ensured so that unnecessary errors can be prevented during researches. The advantages of bee-based sensor are due to its unique characteristics such as simple operation, time-saving and cost effective. This is because a bee hive usually contains up to 30,000 to 70,000 of worker bees (Davidson et al., 2016) and a bee hive usually costs around RM 2000, which means that lot of sniffer bees could be produced. This is potentially cost effective compared to the cost of an analytical instrument with its yearly maintenance expenses usually up to several thousand Ringgit Malaysia. Furthermore, laboratory testing using gas chromatography and/or liquid chromatography require time consuming sample treatment protocols before injection. Analytical technique using liquid chromatography may require hours for *A. paniculata* determination and confirmation.

Previous studies on *A. paniculata* were mostly focused on non-volatile compounds, especially andrographolide, and then detected by high performance liquid chromatography. This study suggests another reliable alternative for *A. paniculata* detection based on its phytochemical profile constructed by volatile compounds. Therefore, the volatile phytochemical profile of *A. paniculata* could be used for quality assurance of herbal related industries.

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APPENDIX A

RESULTS OF KINETIC STUDIES OF VOLATILE MARKER COMPOUNDS

A.1 Kinetic Study of 5,6,7,7α-tetrahydro-4,4,7α-trimethyl-2(4H)-benzofuranone

$$Equation: C_t = \frac{t}{\left(\frac{1}{h}\right) + \left(\frac{t}{C_e}\right)}$$

Time	Observed	Predicted	(y-mean(y))^2	(y_p-y_o)^2
10	133.5345	135.5798	10156.1677	4.1831
15	181.8200	170.3824	2755.4514	130.8171
20	194.0304	195.4706	1622.6317	2.0739
25	207.4390	214.4135	722.1744	48.6427
40	246.1763	250.8828	140.7523	22.1515
50	267.2822	265.9618	1087.0134	1.7435
60	270.4553	277.0636	1306.3126	43.6693
80	306.0444	292.3158	5145.4836	188.4731
110	302.02900	306.1029	4585.5442	16.5966

Time: Sample heating time

Observed: Observed peak area

Predicted: Predicted peak area

[y-mean(y)]^2: [Observed peak area-mean value of observed peak area]^2

(y_p-y_o)^2: (Predicted peak area-Observed peak area)^2

By using Solver function in Microsoft Excel, the values of parameters can be obtained.

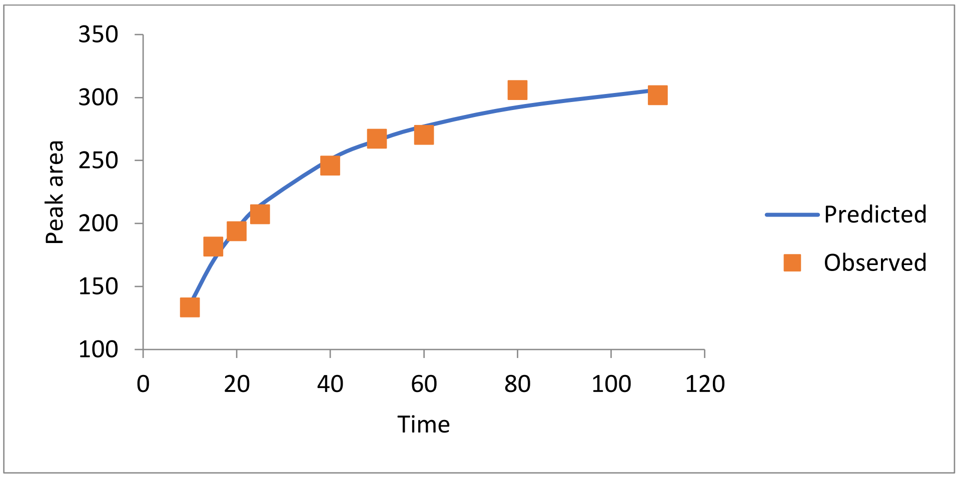
Parameter	Value
h	22.1252
C_e	350.1413

$$R^2 = 1 - \frac{\sum(y_p - y_o)^2}{\sum[y - mean(y)]^2}$$

$$R^2 = 0.9833$$

$$RMSE = \sqrt{\frac{\sum(y_p - y_o)^2}{no.of\ data}}$$

$$RMSE = 7.1364$$



A.2 Kinetic Study of Caryophyllene

$$Equation: C_t = \frac{t}{\left(\frac{1}{h}\right) + \left(\frac{t}{C_e}\right)}$$

Time	Observed	Predicted	$[y\text{-mean}(y)]^2$	$(y_p\text{-}y_o)^2$
10	2050.2065	2060.7405	81268.4535	110.9644
30	2399.2107	2349.6338	4086.7797	2457.8643
40	2356.7056	2391.5424	458.9366	1213.6016
50	2433.4385	2417.4128	9634.5446	256.8219
80	2436.8525	2457.2853	10316.4194	417.4964
90	2339.4956	2464.8138	17.7481	15704.6587
110	2255.1301	2475.8472	6424.4447	48716.0277

Time: Sample heating time

Observed: Observed peak area

Predicted: Predicted peak area

$[y\text{-mean}(y)]^2$: [Observed peak area-mean value of observed peak area]^2

$(y_p\text{-}y_o)^2$: (Predicted peak area-Observed peak area)^2

By using Solver function in Microsoft Excel, the values of parameters can be obtained.

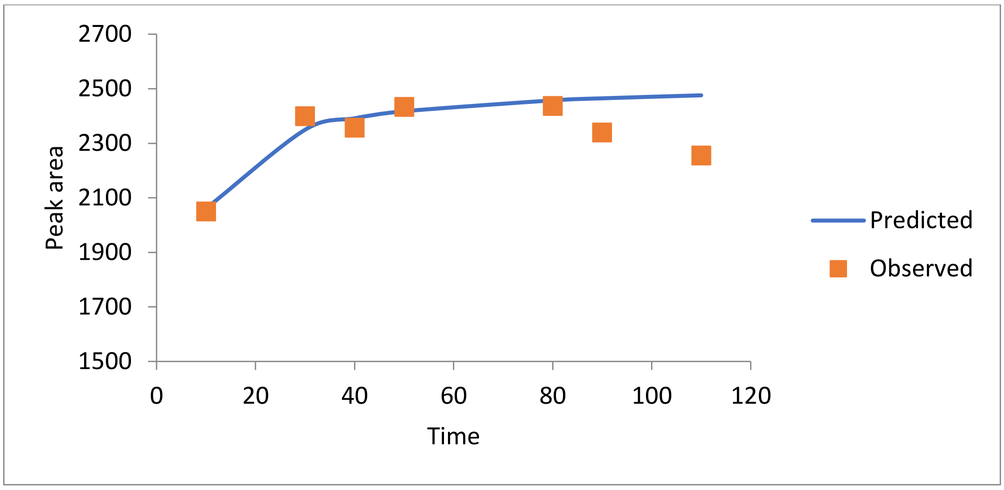
Parameter	Value
h	1117.3641
C_e	2526.7449

$$R^2 = 1 - \frac{\sum(y_p - y_o)^2}{\sum[y - mean(y)]^2}$$

$$R^2 = 0.9579$$

$$RMSE = \sqrt{\frac{\sum(y_p - y_o)^2}{no. of\ data}}$$

$$RMSE = 25.2325$$



A.3 Kinetic Study of β-elemene

$$Equation: C_t = \frac{t}{\left(\frac{1}{h}\right) + \left(\frac{t}{C_e}\right)}$$

Time	Observed	Predicted	[y-mean(y)]^2	$(y_P-y_o)^2$
10	642.5048	659.8107	39622.8778	299.4957
30	853.4323	827.0659	140.9569	695.1819
50	892.4230	871.2358	2587.0700	448.8955
60	885.9645	883.0254	1971.7879	8.6384
80	918.1769	898.2188	5870.1890	398.3233
110	856.8569	911.0443	234.0046	2936.2673

Time: Sample heating time

Observed: Observed peak area

Predicted: Predicted peak area

[y-mean(y)]^2: [Observed peak area-mean value of observed peak area]^2

$(y_P-y_o)^2$: (Predicted peak area-Observed peak area)^2

By using Solver function in Microsoft Excel, the values of parameters can be obtained.

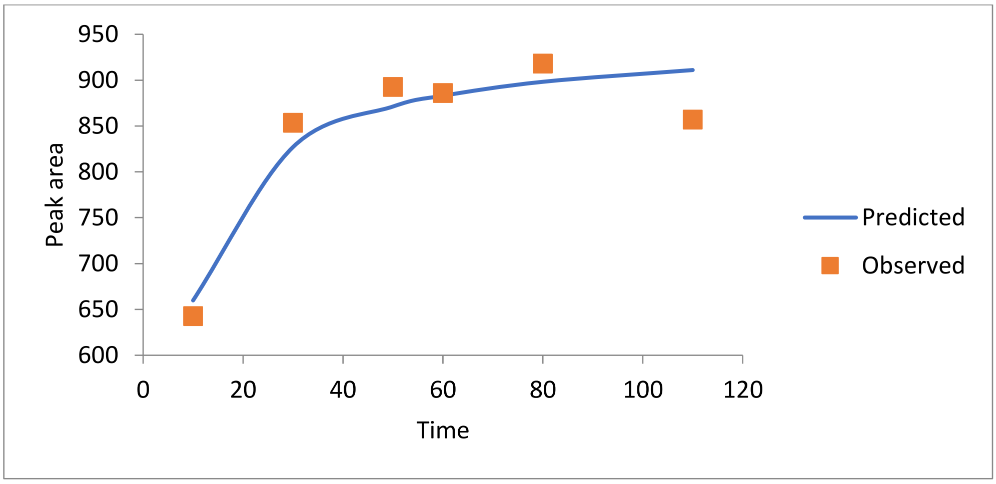
Parameter	Value
h	217.5146
C_e	947.1069

$$R^2 = 1 - \frac{\sum(y_p - y_o)^2}{\sum[y - mean(y)]^2}$$

$$R^2 = 0.9051$$

$$RMSE = \sqrt{\frac{\sum(y_p - y_o)^2}{no.of\ data}}$$

$$RMSE = 28.2454$$



A.4 Kinetic Study of 6,10,14-trimethyl-2-pentadecanone

$$Equation: C_t = C_e[1 - [Fe^{-k_1 t}] - [(1-F)e^{-k_2 t}]]$$

Time	Observed	Predicted	[y-mean(y)]^2	$(y_p\text{-}y_o)^2$
10	61.5333	61.5426	3158.0863	8.45112E-05
15	118.1757	118.1565	0.1984	0.00037
20	128.2121	127.4745	109.8700	0.5440
25	125.8151	129.0082	65.3657	10.1956
40	130.6017	129.3090	165.6752	1.6712
50	128.6495	129.3103	119.2317	0.4366
60	131.1240	129.3103	179.3938	3.2895

Time: Sample heating time

Observed: Observed peak area

Predicted: Predicted peak area

[y-mean(y)]^2: [Observed peak area-mean value of observed peak area]^2

$(y_p\text{-}y_o)^2$: (Predicted peak area-Observed peak area)^2

By using Solver function in Microsoft Excel, the values of parameters can be obtained.

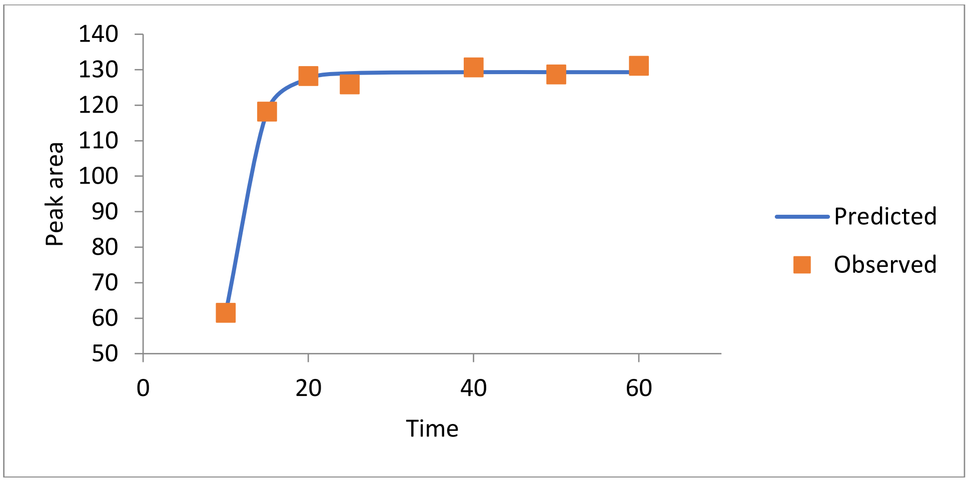
Parameter	Value
C_e	129.31032
F	-18.34602
k_1	135.48671
k_2	0.3608615

$$R^2 = 1 - \frac{\sum(y_p - y_o)^2}{\sum[y - mean(y)]^2}$$

$$R^2 = 0.9958$$

$$RMSE = \sqrt{\frac{\sum(y_p - y_o)^2}{no.of\ data}}$$

$$RMSE = 1.0736$$



A.5 Kinetic Study of Apiol

$$Equation: C_t = C_e[1 - [Fe^{-k_1 t}] - [(1 - F)e^{-k_2 t}]]$$

Time	Observed	Predicted	$[y\text{-mean}(y)]^2$	$(y_p\text{-}y_o)^2$
10	165.9531	165.8233	2946.3597	0.0168
15	217.3516	218.7022	8.3054	1.8243
20	232.8695	229.3131	159.6690	12.6479
30	229.2956	231.8696	82.1229	6.6252
40	237.2076	231.9725	288.1211	27.4060
50	228.9845	231.9767	76.5811	8.9529
60	229.9724	231.9768	94.8464	4.0179

Time: Sample heating time

Observed: Observed peak area

Predicted: Predicted peak area

$[y\text{-mean}(y)]^2$: [Observed peak area-mean value of observed peak area]^2

$(y_p\text{-}y_o)^2$: (Predicted peak area-Observed peak area)^2

By using Solver function in Microsoft Excel, the values of parameters can be obtained.

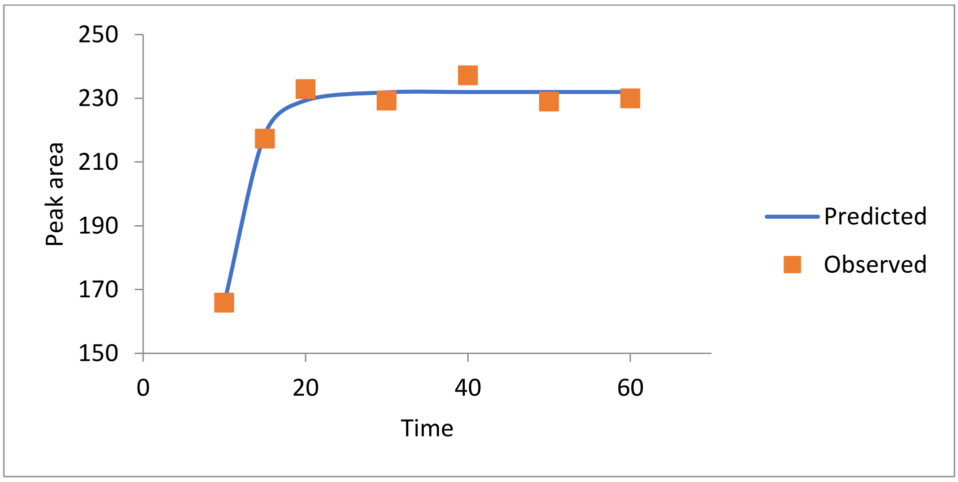
Parameter	Value
C_e	231.9768
F	-6.08222
k_1	135.4867
k_2	0.321225

$$R^2 = 1 - \frac{\sum(y_p - y_o)^2}{\sum[y - mean(y)]^2}$$

$$R^2 = 0.9832$$

$$RMSE = \sqrt{\frac{\sum(y_p - y_o)^2}{no.of\ data}}$$

$$RMSE = 2.9639$$



A.6 Kinetic Study of 3,3-dimethylhexane

$$Equation: C_t = C_e[1 - [Fe^{-k_1 t}] - [(1 - F)e^{-k_2 t}]]$$

Time	**Observed**	**Predicted**	**[y-mean(y)]^2**	**$(y_p\text{-}y_o)^2$**
5	120.3245	110.4987	10082.4868	96.5450
10	127.6258	143.5464	8669.5256	253.4680
15	174.5429	171.0385	2133.8078	12.2807
40	253.1402	252.8815	1050.0298	0.0669
50	274.2744	269.5733	2866.3563	22.1000
80	296.3638	294.6511	5719.5525	2.9333
110	298.8808	302.9627	6106.6071	16.6617

Time: Sample heating time

Observed: Observed peak area

Predicted: Predicted peak area

[y-mean(y)]^2: [Observed peak area-mean value of observed peak area]^2

$(y_p\text{-}y_o)^2$: (Predicted peak area-Observed peak area)^2

By using Solver function in Microsoft Excel, the values of parameters can be obtained.

Parameter	Value
C_e	307.0831
F	0.230468
k_1	135.4867
k_2	0.036811

$$R^2 = 1 - \frac{\sum(y_p - y_o)^2}{\sum[y - mean(y)]^2}$$

$$R^2 = 0.9890$$

$$RMSE = \sqrt{\frac{\sum(y_p - y_o)^2}{no.of\ data}}$$

$$RMSE = 7.5975$$