Chlorophyll extraction from suji leaf (*Pleomele angustifolia* Roxb.) with ZnCl_2 stabilizer

Edia Rahayuningsih¹ · Mukmin Sapto Pamungkas¹ · Muhammad Olvianas¹ · Andreas Diga Pratama Putera¹

Revised: 15 November 2017 / Accepted: 18 December 2017 / Published online: 5 February 2018
© Association of Food Scientists & Technologists (India) 2018

Abstract Suji (*Pleomele angustifolia* Roxb.) leaves are a prominent source of chlorophyll and well-known for their ability to produce green color for food ingredients. However, chlorophyll is susceptible to color degradation at high temperature. Color degradation occurred because *porphyrin* loses magnesium in its ring and it can be avoided by adding zinc. The aim of this work was to investigate the combined effect of independent variables on chlorophyll extraction process using ZnCl_2 as a stabilizer. Suji leaves were blanched with boiling water for 2 min, Zn-chlorophyll synthesis was done by varying concentration of ZnCl_2 , Zn-chlorophyll extraction with ethanol, and UV–Vis spectrophotometry analysis of the final extracted solutions. A full three-level factorial design under response surface methodology was used to obtain the optimum condition of extraction process. The experimental data were analyzed by analysis of variance and fitted with second order polynomial equation. The coefficient of determination (R^2) was found to be 81.99%. The optimum operating conditions were obtained at pH 7, ZnCl_2 concentration of 700 ppm and temperature of 85 °C with desirability value of 1.0000. At the optimum conditions, the total chlorophyll content (TCC) was found to be 47.2975 mg/100 g fresh weight.

Keywords Suji leaves · Chlorophyll · Total chlorophyll content · Optimization

Introduction

Food consumer acceptance of foods is significantly determined by its appearance, especially its color (Sant’Anna et al 2013). In the past, before the accidental invention of widely available and affordable synthetic dye mauveine by Perkin in Germany in 1856, people used natural sources such as stem, bark, leaves, roots and flowers to get various colors (Sinha et al. 2012a). Today, the usage of natural colorants slumped sharply (Shahid et al. 2013). However, the usage of synthetic dyes has been creating several important issues with human health. An article from Arnold et al. (2012) stated that even the artificial food colors are not main cause of attention deficit hyperactivity disorder (ADHD), but they may contribute significantly to some cases. Furthermore, a research by McCann et al. (2007) found that the intake of artificial food color (sodium benzoate) in the diet is increased with the hyperactivity in 3- and 8/9-year-old children in the general population. In terms of physical health, although it has not been tested in human, a research (Amin et al. 2010) concluded that tartrazine and carmoisine have effects in vital organs, namely liver and kidney, not only at higher doses but also at low doses.

With the enhanced public awareness on eco-friendly, health concerns, and non-toxic materials products, synthetic colorants are being actively replaced with the natural ones (Neelwarne and Halagur 2012; Khan et al. 2014). It is well accepted in our society that products made from natural resources are more healthy and safer than the synthetic ones. Such food can be regarded as “functional” food and there is a rising demand on this product due to increasing cost of healthcare, enhancement in life expectancy, and the desire from older to improve their health quality (Siró et al. 2008). The ingredients of this food are also preferred from

✉ Edia Rahayuningsih
edia_rahayu@ugm.ac.id

¹ Department of Chemical Engineering, Universitas Gadjah Mada, Jalan Grafika No. 2 Kampus UGM, Bulaksumur, D. I. Yogyakarta 55281, Indonesia

natural origin than that of the synthetic ones (Plaza et al. 2009). Moreover, fruits and vegetables are particularly rich sources of natural pigments (Swamy et al. 2014).

Suji leaf or narrow-leaf pleomele (*Pleomele angustifolia* Roxb.) is a popular natural source in Indonesia and have been used for various kinds of natural food and beverage dyes. The plant's habitat is rainforest, nearby rivers and streams (WHO 2009) which are easily found in Indonesia. Suji (*Pleomele angustifolia* Roxb.) belongs to Liliaceae family. Its leaf shape is single, elongated, and narrow with length about 17 and 2.5 cm width and has dark green color. In addition, its leaf is not bitter but rather has unique scent (Kinho et al. 2011). It is a rhizomatous, perennial herbaceous plant, forming large climbing shrubs with few ascending branches or small slender trees with weak pendulous twigs. The sessile linear leaves are born in dense rosettes, followed by yellow flowers in terminal racemes to spreading panicles and globose compressed 3-lobed berry. It is widely cultivated as a garden and indoor ornamental plant. Its stems are basic and have only a few branches with smooth and grayish color. The image of suji plants can be seen in Fig. 1a. Traditionally known for its green coloring agent, this plant has 73.25% water content which contains 3773.9 ppm chlorophyll (2524.6 ppm chlorophyll a and 1250.3 chlorophyll b) in its leaves (Aryanti et al. 2016). Both chlorophyll a and chlorophyll b are found in Suji leaves. Chlorophyll a is a complex compound of magnesium and porphyrin that has cyclopentanone ring. Chlorophyll a is insoluble in water but very soluble in ethanol and methanol. The chemical formula of chlorophyll a is $C_{55}H_{72}O_5N_4Mg$. On the other side, chlorophyll b is another form of chlorophyll that helps photosynthesis by absorbing light energy. It is also insoluble in water but very soluble in ethanol and methanol. Its chemical formula is $C_{55}H_{70}O_6N_4Mg$ (Lide 2009). The chemical structures of chlorophyll and its derivatives can be seen in Fig. 1b.

Solid–liquid extraction of chlorophyll usually uses acetone or alcohol solvent (Aryanti et al. 2016). However, chlorophyll is easily degraded in unfavorable pH, temperature, and contact with oxygen or direct light. Some of the derivatives are pheophytin, pheophorbide, pyropheophytin, dan pyropheophorbide. Color degradation occurs because of the substitution of a Mg^{2+} ion with H^+ ion from porphyrin (Koca et al. 2007). On the other side, recent studies show that a more durable chlorophyll color extraction can be conducted with substituting the Mg^{2+} with Zn^{2+} or Cu^{2+} (Senklang and Anprung 2010).

The present work was conducted for optimizing the extraction process of natural pigment from narrow-leaf pleomele (*Pleomele angustifolia* Roxb.) by stabilized aqueous extraction method with the addition of $ZnCl_2$ and to examine the combined effect of experiment parameters such as pH, solvent concentration, and temperature using a

full three-level factorial design in Response Surface Methodology (RSM).

Materials and methods

Materials

Fresh suji leaves were collected from the local market in Magelang, Indonesia. An analytical grade of sodium hydroxide pellet (97 wt%) was obtained from Merck Milipore (Darmstadt, Germany). For other reagents and materials were purchased from CV. Sigma Aldrich (Yogyakarta).

Raw leaves blanching

The suji leaves were washed well with tap water and then boiled for 2 min to soften the leaves. The leaves were then comminuted using commercial blender.

$ZnCl_2$ solution preparation

Solid $ZnCl_2$ was dissolved in aquadest with a certain weight and stored. The unit of $ZnCl_2$ concentration is in ppm, m_{ZnCl_2} is the mass of $ZnCl_2$ added in the solution in miligrams, and $V_{solution}$ is the volume of the solution in litres.

Zn-chlorophyll synthesis

As much as 70 g of blanched suji leaves were mixed with 280 mL of $ZnCl_2$ solution with varied concentration as it can be seen in Table 1. In this stage, the pH was adjusted using NaOH or nitric acid solution. The varied pH is also can be seen in Table 1. The solutions were put in a 2-l autoclave with design pressure up to 30 bars for 15 min at certain reaction temperature as it can be seen also in Table 1.

Zn-chlorophyll extraction with ethanol

As much as 10 mL suji leaves pulp were mixed with 25 mL ethanol 96% in an Erlenmeyer flask. The flask was then isolated using aluminum foil and then put in a shaker waterbath. The process in the shaker waterbath was set in 60 °C with 135 rpm stirring speed for 45 min.

Total chlorophyll content (TCC)

As much as 6 g of the extract from point 2.5 was taken and diluted in 30 mL of acetone. The solution was then mixed well in a magnetic stirrer with 1000 rpm stirring speed for

Fig. 1 **a** Image of suji plants used in this study and **b** structures of chlorophyll and its derivatives

Derivative	Metal	R1	R2
Chlorophyll a	Mg	CH ₃	Phytyl
Chlorophyll b	Mg	CHO	Phytyl
Pheophytin a		CH ₃	Phytyl
Pheophytin b		CHO	Phytyl
Pheophorbide a		CH ₃	H
Pheophorbide b		CHO	H

Table 1 Experimental range and level of independent variables

Independent variables	Range and level		
	+1	0	− 1
pH (X ₁)	11	9	7
[ZnCl ₂], ppm (X ₂)	700	500	300
T, °C (X ₃)	135	110	85

15 min. After this process, as much as 5 mL solution was taken and centrifuged. The clear solution was then analyzed using UV–Vis Spectrophotometer. A Vernier SpectroVis Plus Spectrophotometer (SVIS-PL) was used in this work. The instrument is integrated with Logger Pro® software installed in a computer.

TCC can be deducted from spectrophotometry results in the wavelength of 645 and 663 nm, using equations (Senklang and Anprung 2010; Özkan and Ersus Bilek 2015):

$$CC \left(\frac{\text{mg}}{\text{g fresh weight}} \right) = \frac{((20.2 \times A_{645 \text{ nm}} + 8.02 \times A_{663 \text{ nm}}) \times \text{Dilution factor})}{1000} \quad (1)$$

$$TCC \left(\frac{\text{mg}}{100 \text{ g fresh weight}} \right) = \frac{CC \times 100 \times \text{pulp mass}}{m_{\text{sample}}} \quad (2)$$

where CC is the chlorophyll content in the pulp, A_{645} and $A_{663 \text{ nm}}$ are the absorbances in the wavelength of 645 and 663 nm. The dilution factor in this work is 1, the pulp mass is 6 g, and the m_{sample} is about 350 g, as it can be seen in section spectrophotometry analysis and determination of total chlorophyll content (TCC) and ZnCl₂ solution preparation, respectively.

Design of experiment and statistical analysis

This study aims to optimize several parameters which have an effect in chlorophyll extraction process. Response surface methodology (RSM) was chosen as a tool for optimization. RSM can reduce the number of experiment for evaluating the parameters and their interactions (Sinha et al. 2012b). A full three-level factorial design was used with three independent variables, namely pH, ZnCl₂ concentration and extraction temperature. The dependent variable in this study is total chlorophyll content (TCC), with detail of independent variables and their range and level are written in Table 1.

A second-order polynomial equation was employed to correlate the response (dependent variable) and factors (independent variables). The polynomial equation has a general form which written as follows (Bezerra et al. 2008; Montgomery 2013):

$$Y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{1 \leq i < j \leq k} \beta_{ij} x_i x_j + \varepsilon \quad (3)$$

where Y is the response; x_i and x_j are the factors (i and j is the range from 1 to k); β_0 is the constant coefficient; β_i , β_{ii} , β_{ij} are the coefficients for the linear, quadratic and interaction effect; and ε represents the error. The accuracy of proposed model was assessed using the coefficient of determination (R^2). R^2 has value ranged from 0 to 1, if R^2 close to 1 indicates that the model is more accurate (Vendaraman et al. 2017).

Results and discussions

Identification of derivatives using UV–Vis analysis

The chlorophyll which is extracted from the suji leaves were characterized using UV–Vis spectrophotometer. The selected spectrophotometry results are presented in Fig. 2a–c. UV–Vis spectrophotometry reveals that the produced dyes are composed of several pigments. Figure 2a–c show absorption maxima (λ_{max}) around 430 nm, which belong to β -carotene pigment (Senklang and Anprung 2010). Moreover, cation substitution using Zn^{2+} also confirmed by occurrence of a peak around 655 nm, which possessed by Zn-pheophytin a, Zn-pyropheophytin a, Zn-pheophytin b and Zn-pyropheophytin b (Senklang and Anprung 2010).

Figure 2a–c show the effect of a particular variable when other variables are kept at lowest constant value. In Fig. 2a, sample with run code 19 has higher absorbance value at wavelength of 633 and 645 nm. Meanwhile, the effect of concentration of $ZnCl_2$ gives slightly different results for each run code as can be seen in Fig. 2b. Then, the temperature effect has a significant difference in term of absorbance values as presented in Fig. 2c. The absorbance value of run code 3 has the lowest absorbance value.

Statistical analysis and fitting of second order polynomial equation

This study was performed based on a full three-level factorial design to optimize the extraction process of chlorophyll from suji leaves. Model fitting techniques was performed to obtain the predicted values of TCC using Minitab software and proved to be sufficiently correlated with the experimental values. The experimental and predicted values of TCC are presented in Table 2.

Fig. 2 UV–Vis Spectrophotometry results for the sample with: **a** varied pH treatment, **b** varied $[ZnCl_2]$ concentration treatment and **c** varied temperature treatment

The empirical relationship between response (TCC) and the independent variables (pH, concentration of ZnCl_2 and temperature) has been expressed by the second order polynomial equation.

$$\begin{aligned} TCC\left(\frac{\text{mg}}{100 \text{ gfw}}\right) = & 186.1 - 15.6X_1 - 0.0030X_2 - 1.25X_3 \\ & + 0.459X_1^2 + 0.000125X_2^2 + 0.00184X_3^2 \\ & - 0.00460X_1X_2 + 0.0785X_1X_3 \\ & - 0.000719X_2X_3 \end{aligned} \quad (4)$$

The regression and analysis of variance (ANOVA) were performed to check the adequacy and fitness of the model. ANOVA is a statistical technique that partitions the total variability into its component parts associated with specific sources of variation for the testing of model parameters (Maran et al. 2012; Montgomery 2013). Then, F-test and P value also were used to determine which of the effects in the model are statistically significant. The results of

ANOVA analysis were exhibited in Table 3. As written in Table 3, the P value for proposed model (very small number ~ 0.000) implies statistically significant, due the value lower than the level of significant ($\alpha = 0.05$). The linear effect of temperature also gives a value lower than 0.05, it means that the temperature has a significant effect in extraction process (Sinha et al. 2012b; Özkan and Ersus Bilek 2015). Statistical calculation result for effect of temperature is in a good agreement with UV–Vis spectrophotometry results. A higher extraction temperature significantly decreases the absorbance values. On the other hand, the quadratic and interaction term give P values > 0.05 . Therefore, there is no significant effect toward the extraction process from those terms.

Adequacy of the models

Ensuring the adequacy of predicted values with actual values is important to verify the proposed model.

Table 2 Total chlorophyll content (TCC) results

Run code	pH (X_1)	[ZnCl_2], ppm (X_2)	T, °C (X_3)	Experimental values of TCC, mg/100 g fresh weight	Predicted values of TCC, mg/100 g fresh weight
1	7	300	85	32.91	35.49
2	7	300	110	29.42	21.56
3	7	300	135	4.36	9.93
4	7	500	85	35.00	36.23
5	7	500	110	27.83	18.70
6	7	500	135	3.81	3.47
7	7	700	85	46.43	46.97
8	7	700	110	20.77	25.84
9	7	700	135	7.62	7.02
10	9	300	85	28.12	29.57
11	9	300	110	22.16	19.56
12	9	300	135	5.30	11.85
13	9	500	85	29.11	28.46
14	9	500	110	12.83	14.86
15	9	500	135	9.78	3.55
16	9	700	85	35.02	37.36
17	9	700	110	28.59	20.16
18	9	700	135	3.10	5.26
19	11	300	85	34.88	27.31
20	11	300	110	12.53	21.23
21	11	300	135	27.83	17.44
22	11	500	85	20.00	24.37
23	11	500	110	10.47	14.69
24	11	500	135	6.44	7.31
25	11	700	85	39.28	31.43
26	11	700	110	13.52	18.15
27	11	700	135	8.47	7.18

Table 3 ANOVA analysis and statistical parameters of the model

Source	Effect	Coefficient estimate	Standard error coefficient	DF	Adj. sum of square	Adj. mean square	F-value	P value
Model				9	3416.44	379.60	8.60	0.000
pH	− 3.86	− 1.93	1.57	1	67.01	67.01	1.52	0.235
[ZnCl ₂]	0.62	0.31	1.57	1	1.72	1.72	0.04	0.846
T	− 24.89	− 12.45	1.57	1	2788.64	2788.64	63.18	0.000
pH*pH	3.67	1.83	2.71	1	20.20	20.20	0.46	0.508
[ZnCl ₂]*[ZnCl ₂]	10.00	5.00	2.71	1	150.13	150.13	3.40	0.083
T*T	2.30	1.15	2.71	1	7.93	7.93	0.18	0.677
pH*[ZnCl ₂]	− 3.68	− 1.84	1.92	1	40.69	40.69	0.92	0.350
pH*T	7.85	3.93	1.92	1	185.10	185.10	4.19	0.056
[ZnCl ₂]*T	− 7.19	− 3.59	1.92	1	155.02	155.02	3.51	0.078
R-square	81.99%							

Verification of fitted model can be approached by linear fitting of experimental and predicted values of TCC and normal probability plot. Figure 3a shows the comparison between the experimental and predicted values of TCC. The data points are found close to the straight line and can be justified that there is well correlated between the experimental and predicted values. Based on Fig. 3a, the adequacy and fitness of the model can be quantitatively expressed by coefficient of determination (R^2). The value of R^2 was calculated to be 81.99%, the calculated number implies that 82% of experimental data was compatible with the models. The result indicates that the models used in this research were able to interpret the operating conditions in chlorophyll extraction process using ZnCl₂ as a stabilizer.

Figure 3b depicts the normal probability plot which indicates whether the data are normally distributed, other variables are influencing the response, or outliers exist in the data. The residual gives the variation between the experimental value of a response and the value that is generated from the theoretical model. Highly accurate

model is characterized by its small residual value (Agarry and Ogunleye 2012; Mohajeri et al. 2010). As can be seen in Fig. 3b, the data points are positioned close to the reference line, the outliers data is observed to be minimal compared with the normal data. Therefore, it can be concluded that the data is normally distributed.

Effect of independent variables on the chlorophyll extraction

Chlorophyll extraction from suji leaves was studied with various pH, ZnCl₂ concentration and temperature. This study has a clear objective to find out the optimum extraction conditions. Contour plots were generated from Minitab software and were employed to identify the effect of process variables on the extraction of chlorophyll from suji leaves. The contour plots described the influence of two variables on the process while other variable was maintained at constant value as shown in Fig. 4a–c.

Fig. 3 Linear fitting plot and normal probability plot of standardized residuals for TCC

The combined effect of ZnCl_2 concentration and pH on TCC values of extracted dye is shown in the Fig. 4a. The TCC values are identified to be sensitive with the change of temperature. It is observed that the TCC values increased with increasing the ZnCl_2 concentration as well as decreasing the temperature. From the contour plot of Fig. 4a, the maximum TCC value can be obtained at ZnCl_2 concentration of 700 ppm and temperature of 85 °C. A lower temperature condition is favorable condition for extraction process. It has been proved by a research from Kong et al. (2014), which stated there is a decline of chlorophyll yield with further increase of extraction temperature in ultrasound-assisted extraction process.

Figure 4b illustrates the effect of different level of pH and temperature on TCC values. Both of variables in Fig. 4b have a strong effect on the extraction process. The contour plot of Fig. 4b shows the maximum TCC value is achieved at pH 7 and temperature of 85 °C. This result is in an agreement with the findings of Koca et al. (2007). If the extraction process is conducted in acidic solution, it will trigger the replacement of $[\text{Mg}^{2+}]$ in chlorophyll with two $[\text{H}^+]$ ions. Özkan and Ersus Bilek (2015) in their study of chlorophyll extraction from spinach also found that $\text{pH} > 7$ also will not significantly affect the TCC. A higher pH

leads to accelerate the $\text{Zn}(\text{OH})_2$ formation, which cannot form complexes with chlorophyll derivatives (Özkan and Ersus Bilek 2015).

The contour plot in Fig. 4c shows the combined effect of pH and ZnCl_2 concentration on the total chlorophyll content. Contour plot of Fig. 4c has a minimum-type of graph, it reveals there is an interaction between pH and ZnCl_2 concentration. To obtain maximum TCC value, it is suggested to conduct the experiment at pH 7 and ZnCl_2 concentration of 700 ppm.

Selection of optimum conditions

The numerical optimization provides the exact value for an optimal experimental set up. The influence of each operational condition like pH, ZnCl_2 concentration, and temperature with the response (TCC) was examined to get best local maximum. The possible results of numerical optimization are in the range of process variables and a maximum value of response. A level of pH within range of 7–11, the ZnCl_2 concentration within range of 300–700 ppm and the level of temperature within range of 85–135 °C were set as constraints to achieve maximum desirability. The desirability value has a scale between 0

Fig. 4 Contour plots showing the effects of independent variables on the TCC

(completely undesirable response) and 1 (fully desired response). Applying the methodology for optimization, the local maximum of independent variables was obtained and it indicates the pH 7, ZnCl_2 concentration of 700 ppm and temperature of 85 °C give 47.2975 mg/100 g fw of TCC with the desirability value of 1.0000.

Conclusion

In this research, a full three-level response surface design is proved to be effective and reliable for optimizing chlorophyll extraction process from suji leaves. The independent variables showed a strong effect on extraction process. The contour plots were employed to assess the combined effect of three independent variables (pH, ZnCl_2 concentration and temperature) on the response (TCC). A second order polynomial equation was used to correlate response and independent variables. The coefficient of determination (R^2) of 81.99% and normal probability plot ensures the adequacy of proposed model with experimental results. The optimum conditions (pH 7, ZnCl_2 concentration of 700 ppm and temperature of 85 °C) of chlorophyll extraction yields TCC of 47.2975 mg/100 g fw.

Acknowledgements Highly appreciation to the Department of Chemical Engineering Universitas Gadjah Mada for the research facilities provided to run this experiment.

References

- Agarry SE, Ogunleye OO (2012) Factorial designs application to study enhanced bioremediation of soil artificially contaminated with weathered bonny light crude oil through biostimulation and bioaugmentation strategy. *J Environ Prot* 3:748–759. <https://doi.org/10.4236/jep.2012.38089>
- Amin KA, Abdel Hameid H, Abd Elsttar AH (2010) Effect of food azo dyes tartrazine and carmoisine on biochemical parameters related to renal, hepatic function and oxidative stress biomarkers in young male rats. *Food Chem Toxicol* 48(10):2994–2999. <https://doi.org/10.1016/j.fct.2010.07.039>
- Arnold LE, Lofthouse N, Hurt E (2012) Artificial food colors and attention-deficit/hyperactivity symptoms: conclusions to dye for. *Neurotherapeutics* 9:599–609. <https://doi.org/10.1007/s13311-012-0133-x>
- Aryanti N, Nafiunisa A, Willis FM (2016) Ekstraksi dan Karakterisasi Klorofil dari Daun Suji (*Pleomele Angustifolia*) sebagai Pewarna Pangan Alami. *Jurnal Aplikasi Teknologi Pangan* 5(4):129–135. <https://doi.org/10.17728/jatp.183129>
- Bezerra MA, Santelli RE, Oliveira EP, Villar LS, Escalera LA (2008) Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta* 76:965–977. <https://doi.org/10.1016/j.talanta.2008.05.019>
- Khan AA, Iqbal N, Adeel S, Azeem M, Batool F, Bhatti IA (2014) Extraction of natural dye from red calico leaves: gamma ray assisted improvements in colour strength and fastness properties. *Dyes Pigments* 103:50–54. <https://doi.org/10.1016/j.dyepig.2013.11.024>
- Kinho J, Arini, DID, Halawane, J, Nurani L, Halidah Kafiari Y, Karundeng MC (2011) *Tumbuhan obat tradisional di Sulawesi Utara*. 2nd edn. Manado: Indonesian Ministry of Forestry. Available at: <http://forda-mof.org/files/TumbuhanObatTradisionalSulawesi.pdf>
- Koca N, Karadeniz F, Burdurlu HS (2007) Effect of pH on chlorophyll degradation and colour loss in blanched green peas. *Food Chem* 100(2):609–615. <https://doi.org/10.1016/j.foodchem.2005.09.079>
- Kong W, Liu N, Zhang J, Yang Q, Hua S, Song H, Xia C (2014) Optimization of ultrasound-assisted extraction parameters of chlorophyll from *Chlorella vulgaris* residue after lipid separation using response surface methodology. *J Food Sci Technol* 51:2006–2013. <https://doi.org/10.1007/s13197-012-0706-z>
- Lide DR (2009) CRC handbook of chemistry and physics, 90th edn. CRC Press, Boca Raton
- Maran JP, Manikadan S (2012) Response surface modeling and optimization of process parameters for aqueous extraction of pigments from prickly pear (*Opuntia ficus-indica*) fruit. *Dyes Pigments* 95:465–472. <https://doi.org/10.1016/j.dyepig.2012.06.007>
- McCann D, Barret A, Cooper A, Crumpler D, Dalen L, Grimshaw K, Kitchin E, Lok K, Porteous L, Prince E, Sonuga-Barke E, Warner JO, Stevenson J (2007) Food additives and hyperactive behaviour in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial. *Lancet* 370:1560–1567. [https://doi.org/10.1016/S0140-6736\(07\)61306-3](https://doi.org/10.1016/S0140-6736(07)61306-3)
- Mohajeri L, Aziz HA, Isa MH, Zahed MA (2010) A statistical experiment design approach for optimizing biodegradation of weathered crude oil in coastal sediments. *Bioresour Technol* 101:893–900. <https://doi.org/10.1016/j.biortech.2009.09.013>
- Montgomery DC (2013) Design and analysis of experiments, 8th edn. Wiley, Hoboken
- Neelwarne B, Halagur SB (2012) Red beet: an overview In: Red beet biotechnology: food and pharmaceutical applications, Springer, New York, pp 1–43. <https://doi.org/10.1007/978-1-4614-3458-0>
- Özkan G, Ersus Bilek S (2015) Enzyme-assisted extraction of stabilized chlorophyll from spinach. *Food Chem* 176:152–157. <https://doi.org/10.1016/j.foodchem.2014.12.059>
- Plaza M, Herrero M, Cifuentes A, Ibáñez E (2009) Innovative natural functional ingredients from microalgae. *J Agric Food Chem* 57(16):7159–7170. <https://doi.org/10.1021/jf901070g>
- Sant'Anna V, Gurak PD, Ferreira Marczak LD, Tessaro IC (2013) Tracking bioactive compounds with colour changes in foods—a review. *Dyes Pigments* 98(3):601–608. <https://doi.org/10.1016/j.dyepig.2013.04.011>
- Senklang P, Anprung P (2010) Optimizing enzymatic extraction of Zn-chlorophyll derivatives from pandan leaf using response surface methodology. *J Food Process Preserv* 34:759–776. <https://doi.org/10.1111/j.1745-4549.2009.00393.x>
- Shahid M, Shahid-Ul-Islam, Mohammad F (2013) Recent advancements in natural dye applications: A review. *J Clean Prod* 53:310–331. <https://doi.org/10.1016/j.jclepro.2013.03.031>
- Sinha K, Saha PD, Datta S (2012a) Response surface optimization and artificial neural network modeling of microwave assisted natural dye extraction from pomegranate rind. *Ind Crops Prod* 37(1):408–414. <https://doi.org/10.1016/j.indcrop.2011.12.032>
- Sinha K, Saha PD, Datta S (2012b) Extraction of natural dye from petals of Flame of forest (*Butea monosperma*) flower: process optimization using response surface methodology (RSM). *Dyes Pigments* 94:212–216. <https://doi.org/10.1016/j.dyepig.2012.01.008>
- Siró I, Kápolna E, Kápolna B, Lugasi A (2008) Functional food. Product development, marketing and consumer acceptance—a

- review. *Appetite* 51(3):456–467. <https://doi.org/10.1016/j.appet.2008.05.060>
- Swamy GJ, Sangamithra A, Chandrasekar V (2014) Response surface modeling and process optimization of aqueous extraction of natural pigments from Beta vulgaris using Box-Behnken design of experiments. *Dyes Pigments* 111:64–74. <https://doi.org/10.1016/j.dyepig.2014.05.028>
- Vedaraman N, Sandhya KV, Charukesh NRB, Venkatakrishnan B, Haribabu K, Sridharan MR, Nagarajan R (2017) Ultrasonic extraction of natural dye from Rubia Cordifolia, optimisation using response surface methodology (RSM) & comparison with artificial neural network (ANN) model and its dyeing properties on different substrates. *Chem Eng Process* 114:46–54. <https://doi.org/10.1016/j.cep.2017.01.008>
- WHO (2009) Medicinal plants in Papua New Guinea, WHO series. Manila: WHO Press. Available at: <http://apps.who.int/medicine/docs/documents/s21363en/s21363en.pdf>