**Recover rutin from *Labisia pumila* extract: percentage of methanol and volume of elution using solid phase extraction**

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**Abstract**

The method of heat reflux was used to extract the leaves of *Labisia pumila* var. *Alata* in a methanolic solvent system (60 %)*.* Sample clean-up was carried out on the crude extract to obtain high yield of rutin using a C18 reversed phase solid phase extraction. The volume of eluent required for rutin elution was found to decrease with the increase of methanol concentration (20-100 %). The recovery of rutin was increased using the 20-80 % methanol system, and slightly decreased at the 100 % methanol system. Approximately, 70 % of rutin could be recovered using the 80 % methanol system. This solvent system also shows the lowest distance (9.44 MPa1/2) in the Hansen solubility. The optimum rutin rich fraction achieved 3.96 mg/g fraction representing 4-fold increment from crude extract. The increment was also noticed for the antioxidant capacity in term of scavenging activity which was 2 times higher than crude extract, but 6 times lower than standard rutin. A portion of water (20 %) in the eluent could slightly improve the yield of rutin rich fraction. The role of water is important for the elution of glycosylated flavonol (rutin) compared to the eluent system of 100 % methanol.

**Keywords**: rutin; *Labisia pumila*; solid phase extraction; aqueous methanol; scavenging activity

**1. Introduction**

Rutin (3′,4′,5,7-tetrahydroxyflavone-3-rutinoside) is one of the attractive plant-based glycosyl flavonoids because of its remarkable pharmacological activities.1 This glycoside consists of its aglycone, quercetin and two sugar moieties; glycose and rhamnose, sometimes it is called as quercetin-3-O-rutinoside. Quercetin usually coexists with rutin in plants and in intestine after consumed and hydrolyzed by gastrointestinal microflora.2 They are excellent sources of pharmaceutical products for phytotherapy nowadays.3 The medical benefits of rutin can be seen from its wide application in more than 130 therapeutic formulations worldwide.4-6 Indeed, the demand for natural rutin is in the increasing trend, in line with the increase of scientific evidence on the beneficial effects of rutin.

Numerous studies have been extensively carried out to investigate the extraction methods for high yield of rutin from plant samples. This includes traditional and advanced technological methods as reviewed by Chua.1 Reflux extraction is kwon for its simpler set-up, easy for operation, time and cost effective method, especially for phytochemical extraction from plant samples with an appropriate control on the use of heat and duration of refluxing. Sample clean-up process is usually followed up after extraction for highly complex mixture of samples. Solid phase extraction (SPE) is a commonly employed method for sample clean-up process. The principal of SPE strongly depends on the physicochemical property of its stationary phase, ranging from highly polar to non-polar packing materials in a column. Usually, reversed phase SPE column is used to remove plant impurities such as sugars, proteins and metals prior to analyses in order to prevent matrix interference and improve data reliability.7-9 Previous results indicated that rutin could have the highest adsorption capacity in C18 reversed phase.10 This long organosilyl ligand phase has higher carbon percentage and lower polarity in chemically bonded packing materials compared to C8 and C4.

Solvent is the dominant factor not only for extraction, but also for SPE fractionation of plant samples. It acts as a carrier to deliver phytochemicals into medium. The choice of solvent usually follows the principle of “like dissolves like” which explains that solvent with the polarity value near to the polarity of target compound is likely to be dissolved better and *vice versa*. Since 1924, rutin has been extracted by using polar alcoholic solvents such as ethanol and methanol in many studies.11-13 Pure organic solvent may not be an effective solvent system because mostly 50-60 % of alcoholic solvent could produce the highest yield of rutin from buckwheat.14, 15 A portion of water would enhance the efficiency of extraction by increasing the diffusion of extractable polyphenols through plant tissues.16 Aqueous solvent can increase the polarity of the solvent system for better separation of rutin from complex pharmaceutical and plant samples.17-19

This study focused on the investigation of rutin recovery from the crude extract of *Labisia pumila* var. *Alata* using different percentages of methanol as the eluent in C18 reversed phase SPE. Rutin is likely to be one of the key compounds contributing to the previously reported pharmacological activities of this plant. Plant extract rich in rutin is believed to enhance these biological effects. The presence of rutin was detected by high sensitivity and reliability analytical technique, namely multiple reaction monitoring. This target compound analysis was used to monitor the elution of rutin collected from SPE cartridges, even though rutin was present in trace amount. The rutin-rich fraction was then analyzed for its antioxidant capacity by using colorimetric method for free radical scavenging activity. The finding of this study is important for natural rutin recovery from plant-based samples and wastes, particularly on the effect of methanol concentration in SPE system.

**2. Materials and Methods**

***2.1 Chemicals and plant leaves***

The standard chemical of rutin (97 %) was purchased from Acros Organics (Pittsburgh, USA). HPLC-grade of methanol, n-hexane, ethyl acetate, hydrochloric acid and formic acid were obtained from Merck (Darmstadt, Germany). Sodium carbonate and aluminium chloride were purchased from Fisher Scientific (Pittsburgh, USA). 1-1 diphenyl-2-picrylhydrazyl (DPPH) and L-ascorbic acid were bought from Sigma-Aldrich (St. Louis, MO). Deionised water was generated from Barnstead NANOpure Diamond water purification system (State of Illinois, USA) at 18.2 MΩ-cm resistivity. C18 ec octadecyl-modified silica cartridges (Chromabond, 1000 mg, 6 mL) were bought from Macherey-Nagel (Hoerdt, France). The leaves of *Labisia pumila* var. *Alata* was purchased from Fidea Resources (Selangor, Malaysia). The leaves were rinsed and dried in an oven at 45 oC for 3 days until constant weight. The dried leaves were ground to approximately 2-5 mm for further experimental works.

***2.2 Plant sample extraction***

The dried and ground leaves (150 mg) of the plant were pre-treated with hexane to remove fatty substances by sonication at 30 oC for 15 min. The defatted filtrate was then extracted with 100 mL of 60 % methanol in a reflux system at 75 oC for an hour. The supernatant was collected after extraction and the remaining filtrate was extracted again with another fresh solvent under the similar extraction conditions in order to ensure complete extraction for rutin from the plant leaves. The supernatant was combined and dried to a constant weight by a rotary evaporator at 55 oC. The plant crude extract (27.3 mg) was stored at -20 oC freezer for the subsequent analysis.

***2.3 Solid phase extraction of rutin***

A reversed phase solid phase extraction was carried out to fractionate rutin from the plant crude extract using the principle of column chromatography. The C18 ec cartridge with 14 % of carbon content and 45 µm of particle size was used for rutin fractionation. The cartridge was preconditioned before use according to the instruction of manufacturer. A 1 mL of crude extract (60 mg/mL) was prepared and loaded onto the preconditioned cartridge and eluted with different polarities of methanol concentration at a flow rate of 0.25 mL/min. The eluent system consisted of methanol and water ranging from 20 to 100 % methanol. Each fraction consisted of 1 mL of eluent and screened for rutin detection using an Ultra Performance Liquid Chromatography integrated with tandem Mass Spectrometer (UPLC-MS/MS). The fractions containing rutin would be combined and dried *in vacuo* for rutin quantitation using similar analytical tool, UPLC-MS/MS. The volume of eluent required for rutin fractionation was monitored until completion. The fraction containing rutin was dried and determined for its concentration.

***2.4 UPLC-MS/MS***

The analytical UPLC (Waters Acquity, Milford, MA) system was coupled with a triple quadrupole-linear ion trap tandem mass spectrometer (Applied Biosystems 4000 Q TRAP; Life Technologies Corporation, Carlsbad, CA) with an electrospray ionization source. A C18 reserved phase Acquity column (150 x 4.6 mm, 1.7 µm) protected by a guard column was used throughout this study. The mobile phase was a binary solvent system consisting of solvent A (water with 0.1% formic acid) and solvent B (CH3CN). The UPLC gradient was: 0–3 min, 10% B; 3–8 min, 10–90% B; 8–12 min, 90% B; 12–13 min, 90–10% B; 13–15 min, 10% B for final washing and equilibration of the column for the next run. The flow rate was 0.25 mL/min and the injection volume was 5 µl. All samples were filtered with 0.2 µm nylon membrane filter prior to injection. The negative scan mode of multiple reaction monitoring with two transition ions (m/z 609/301 and m/z 609/151) was used for rutin screening and quantitation. The calibration curve was prepared by using a serial of standard rutin solutions with different concentrations from 0.1 to 1.0 ppm. The capillary and voltage of the ion source were maintained at 400 oC and -4.5 kV, respectively. All other parameters were as follows: nitrogen was used as ion source gas for nebulisation, 40 psi; for drying solvent, 40 psi; curtain gas, 10 psi; collision gas, high; declustering potential, -40 V, and collision exit energy, -10 V. The scan rate was 1000 amu/s. Data acquisition and data processing were performed using Analyst 1.4.2.

***2.5 Free radical scavenging activity by DPPH assay***

The scavenging activity of the fractions was compared to crude extract, standard rutin, standard ascorbic acid by using DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. A 5 mL of DPPH (0.1 mM) in methanol was mixed with 200 µL of samples with different concentrations. After 30 minutes of incubation in a dark place, the absorbance of the solution was measured by using a UV-Vis spectrophotometer (Shimadzu UV-1800, Tokyo, Japan) at 517 nm. The reagent solution without sample was used as blank. The percentage of inhibition was calculated from Equation (1). The inhibitory activity at 50 % (IC50) was determined from the curve constructed by Equation (1). The DPPH assay was carried out in triplicate for all samples.

Inhibition (%) = [(Ac - As)/Ac] x 100 (1)

Where: Ac = absorbance of blank,

As = absorbance of sample or standard

**3. Results and Discussion**

***3.1 Reflux extraction for plant crude extract***

A reflux system was set up to extract phytochemicals from the leaves of *L. pumila* in 60% methanol. This continuous solvent evaporation and condensation process of solid-liquid extraction technique is an effective method for phytochemical extraction because of the ease of operation at reasonable cost. The solvent system (60% methanol) was chosen based on the finding of previous study conducted by the same group of researchers who did the extraction for *L. pumila.*20 Alcoholic solvent is well known as the solvent of choice for phytochemical extraction, especially for polyphenols and terpenoids.21 Since methanol is more polar than ethanol, methanol is likely to be better in penetrating plant cellular membranes than ethanol for phytochemical extraction.22 However, methanol might not be the solvent of choice for those researchers who would like to perform cell-based assays because of high cytotoxicity of methanol.23 Pure methanol was also found to be less effective aqueous methanol.24 In particular, rutin is a glycosylated quercetin with two sugar moieties. Therefore, a portion of water was necessary to further increase the solvent polarity for the enhancement of extraction efficiency.25 According to Ammar et al.,26 the type of solvent and the method of extraction are the most important factors for the extraction of bioactive compounds from plant samples. Approximately, 18.2 % of crude extract was obtained in the present study. The result was found to be higher than the extraction yield of the similar herbal plant in 100% ethanol (6.0%) and 100% water (13.4%) reported by Azrie et al..27

In comparison with the rutin content in the crude extract, the present study produced the extract with the rutin concentration, 1.41 mg/g extract which was comparable to the previous results ranged from 0.46 – 2.12 mg/g extract28 and 0.73 – 2.79 mg/g extract.29 The result of this study was also found to be higher than the findings of Karimi et al..30-32 A broad range of rutin content (4.60-116.85 µg/g extract) was observed, although they are from the similar research group. Hence, the variance in rutin content is not only attributed to the solvent system, but also the extraction conditions such as temperature, time and extraction method, as well as the variety and maturity stage of the herb.

***3.2 Reversed Phase Column Fractionation for Rutin***

A C18 reversed phase column was used for rutin fractionation from the plant crude extract using the eluent system of methanol at different concentrations (20-100 % methanol). It was found that the total volume of eluent (line bar) and the volume required (dot bar) for rutin elution were varied from 20-100 % of methanol as presented in the primary axis of Figure 1. Similarly, the concentration of rutin eluted from the column also varied at different solvent systems as presented in the secondary axis of Figure 1. The eluent system of 20 % methanol required the largest volume of solvent to be discarded before rutin elution, as well as the largest volume of solvent required for rutin elution from the SPE column, but the lowest recovery of rutin (~18 %) in the fractionation. As the concentration of methanol was increased, the capacity ratio (k’) would be decreased which means that shorter retention time of eluent in the stationary phase for faster elution. Therefore, rutin must be highly soluble in that particular eluent system, so that it can follow the eluent flowing out from the packed column quickly.

**Figure 1.** Total volume of eluent (line bar) and volume of eluent containing rutin (dot bar) at the primary axis, and rutin fractionated (line graph) from the methanolic system of solid phase extraction at the secondary axis

As the concentration of methanol was increased, the volume of eluent that required for rutin elution was reduced significantly. Rutin was detected at the first 1-mL of elution for the solvent systems of 80 and 100 % methanol. Both the solvent systems used the smallest volume (3 mL) for complete rutin elution and 80 % methanol produced the higher recovery 69.5 %. This percentage was comparable to the recovery of rutin in the solvent system of 60 % methanol. However, 60 % methanol was not effective enough because higher volume of eluent was required for rutin elution. Therefore, the affinity of rutin in the 80 % methanol system appeared to be the highest among the methanolic systems. This is because rutin could be eluted from the column at the smallest volume of solvent and the highest recovery. A small portion of water (20 %) was required for the optimum rutin elution. The 100% methanol system seems not to be the most effective solvent system for rutin elution. The observation was not in good agreement with the findings of Bulgarian researchers who reported 100 % methanol could recover the highest content of rutin, isoquercitrin, narcissin and astragalin using C18 SPE column for the European *Bupleurum* species, namely *B. baldense* Turra and *B. affine* Sadler.33 Nevertheless, the recovery of the flavonoids including rutin using 75 % methanolic eluent still exhibited the second highest results which were very close to the data of 100 % methanol.

Based on the Hansen solubility parameters, rutin appears to be well dissolved in the eluent system of 80 % methanol. This is because rutin displays the lowest distance (9.44 MPa1/2) from the mass center of Hansen sphere in this solvent system (Table 1). The lower distance can provide better miscibility of rutin in the solvent system. The distance is calculated by the solvent blend formulation which is based on the square root of the sum of the difference between partial cohesive energy of solvent and rutin.34 The energy consists of dispersion, hydrogen bonding and polar bonding which can be estimated from the group contribution method.35 The Hansen solubility parameters can describe the solubility of solute in solvent better than Hildebrand solubility and log P value.36 Hildebrand parameter could only describe the solubility of solute in non-polar and non-hydrogen bonding solvent, whereas the one dimensional partition coefficient which is expressed as log P is very limited for ionizable compound like rutin.35 Rutin has many hydroxyl groups in which their protons are easily released in aqueous based solvents (alcohol and water). In the present study, only half of the rutin content was recovered under the water free eluent. The crucial requirement for a small portion of water has also been highlighted in polyphenol extraction16 and SPE fractionation in many studies.17-19 Most probably, the preference of sugar moiety from rutin in the aqueous medium, even though its aglycone, quercetin is highly soluble in methanol.

**Table 1**. Rutin solubility in different solvent systems based on Hansen solubility parameters

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Hansen solubility parameters (MPa1/2)** | | | |
| **Methanol (%)** | **Dispersion D)** | **Polar bonding (P)** | **Hydrogen bonding (H)** | **Distance\*** |
| Rutin | 19.30 | 16.10 | 25.40 | - |
| Water | 15.50 | 16.00 | 42.30 | 18.53 |
| 20 | 15.34 | 15.26 | 38.30 | 15.16 |
| 40 | 15.18 | 14.52 | 34.30 | 12.23 |
| 60 | 15.02 | 13.78 | 30.30 | 10.13 |
| 80 | 14.86 | 13.04 | 26.30 | 9.44 |
| 100 | 14.70 | 12.30 | 22.30 | 10.43 |



Where s denotes for solvent and r denotes for rutin

By considering the effectiveness of fractionation, 80 % methanol could produce about 60.66 µg rutin in a gram of fraction in a milliliter of eluent which was the highest achievement among the other solvent systems (13.84-60.66 µg rutin/g fraction/mL eluent). This information is very important, especially for those researchers who would like to recover rutin by using the minimum level of solvent consumption. The performance of 100 % methanol was found to be the second highest, which was about 52.27 µg rutin in a gram of fraction in a milliliter of eluent. The content of rutin was increased from 0.85 ± 0.16 mg/g plant leaves (0.08 %w/w) or 1.41 ± 0.54 mg/g crude extract (0.14 %w/w) after reflux extraction, to 3.96 ± 0.39 mg/g fraction (0.40 %w/w) after fractionation using 80 % methanol as the eluent. The increment was about 2-fold after extraction and 4-fold after fractionation. The quantitation of rutin was measured by UPLC-MS/MS under the multiple reaction monitoring method of two transition ions such as m/z 609>301 and m/z 609>151 at negative ion mode as shown in Figure 2.



m/z 609>301

m/z 609>151

**(b)**



**(a)**

**Figure 2**. (a) Two transition ions of rutin peaks in multiple reaction monitoring and (b) mass fragmentation of rutin at the negative ion mode

***3.3 Scavenging activity of rutin fraction***

The quality of rutin fraction was evaluated based on its scavenging activity using DPPH assay compared to standard chemicals such as rutin and ascorbic acid. The free radicals generated from DPPH were scavenged by antioxidants in a concentration dependent manner. This method measured the colour change based on the reduction of purple-coloured free radical DPPHradical dot to yellow-coloured 2,2-diphenyl-1-picrylhydrazine (DPPH–H). The colour density was recorded by a UV-Vis spectrophotometer at 517 nm. The results showed that IC50 for standard rutin and ascorbic acid was 122 and 84 ppm, respectively. The lower IC50 value indicates the lower amount of sample required to inhibit 50 % of free radicals which means the sample has higher scavenging activity. The antioxidant capacity of standard rutin is comparable to ascorbic acid which is a well-known compound for its antioxidant activity. The IC50 of rutin fraction from the 80 % methanol eluent system was 800 ppm which was 6 times lower scavenging activity than standard rutin, but almost 2 times higher scavenging activity than its crude extract (Table 2). Therefore, reversed phase fractionation increased the recovery of rutin in the plant sample, as well as improved its antioxidant capacity. The observation also explains that rutin could be the major radical scavenger. The increase of rutin content in the fractionated sample was found to increase its scavenging activity significantly.

**Table 2**. Scavenging activity of standard chemicals and plant samples at 50% inhibition

|  |  |
| --- | --- |
| **Sample** | **IC50 (ppm)** |
| Standard ascorbic acid | 84 |
| Standard rutin | 122 |
| Crude extract | 1500 |
| 80 % methanol rutin fraction | 800 |

**4. Conclusion**

The detection of rutin has been reported by many investigators in *L. pumila* recently. It could be the prominent flavonol glycoside in the herbal plant which contributes to the significant pharmacological activities. Therefore, this study investigated the effects of methanol concentration for rutin recovery in SPE. A reserved phase SPE system could recover about 70 % of rutin from the crude extract of *L. pumila* var. *Alata* using 80 % methanol as the eluent. The rutin-riched fraction was found to exhibit higher scavenging activity than crude extract, but the value was lower than standard rutin.

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**References**

1. L. S. Chua, *J. Ethnopharmacol*. **2013**, *150,* 805-817.

2. G. Chen, H. Zhang, J. Ye, *Anal. Chim. Acta* **2000**,*423*, 69-76.

3. Y. Yang, F. Zhang, *Ultrason. Sonochem*.**2008**, *15,* 308-313.

4. J. E. F. Reynolds, *Martindale-The Extra Pharmacopoeia,* 31st ed., The Royal Pharmaceutical Society, Council of the Royal Pharmaceutical Society of Great Britain, London, **1996**, pp. 1679-1680.

5. W. Q. Sun, J. F. Sheng, *Handbook of Natural Active Constituents,* Chinese Medicinal Science and Technology Press, Beijing, **1998**, pp. 2240-2316.

6. I. Erlund, T. Kosonen, G. Alfthan, J. Maenpaa, K. Perttunen, J. Kenraali, J. Parantainen, A. Aro, *Eur. J. Clin. Pharmacol*. **2000**, 56, 545-553.

7. S. Y. Yoon, W. J. Choi, J. M. Park, J. W. Yang, *Biotechnol. Tech*. **1997**, 11, 553-556.

8. E. Aehle, S. R. L. Grandic, R. Ralainirina, S. Baltora-Rosset, F. Mesnard, C. Prouillet, J. C. Maziere, M. A. Fliniaux, *Food Chem*. **2004**, 86, 579-585.

9. J. Wang, F. A. Wu, H. Zhao, L. Liu, Q. S. Wu, *Afr. J. Biotechnol*. 2008, **7**, 2147-2155.

10. B. Buszewski, S. Kawka, Z. Suprynowicz, T. Wolski, *J. Pharm. Biomed. Anal.* **1993**, *11,*211-215.

11. C. E. Sando, J. U. Lloyd, *J. Biol. Chem*.**1924**, 737-745.

12. H. F. Koones, N. J. Clifton, *Extraction of rutin,* United States Patent Office: 2,450,555, 5 Oct 1948.

13. F. Fathiazad, A. Delazar, R. Amiri, S. D. Sarker, *Iranian J. Pharm. Res*.**2006**, *5,* 222-227.

14. S. Kreft, M. Knapp, I. Kreft, *J. Agr. Food Chem*. **1999**, *47,* 4649-4652.

15. K. H. Kim, K. W. Lee, D. Y. Kim, H. H. Park, I. B. Kwon, H. J. Lee, *Bioresour. Technol*. **2005**, 96, 1709-1712.

16. E. Altiok, D. Baycin, O. Bayraktar, S. Ulku, *Sep. Purif. Technol.* **2008***, 62,* 342-348.

17. J. Dai, R. J. Mumper, *Molecules,* **2010***,* *15,* 7313-7352.

18. Z. Legnerova, D. Satınsky, P. Solich, *Anal. Chim. Acta* **2003**, 497, 165-174.

19. B. Buszewski, S. Kawka, T. Wolski, *Chromatographia* **1993**, *35*, 311-316.

20. L. S. Chua, N. A. Latiff, S. Y. Lee, C. T. Lee, M. R. Sarmidi, R. A. Aziz, *Food Chem*. **2011**, 127, 1186-1192.

21. A. Pandey, S. Tripathi, *J. Pharmacog. Phytochem*. **2014**, *2,* 115-119.

22. G. X. Wang, *Vet. Parasitol.* **2010**, *171*, 305-313.

23. P. Tiwari, B. Kumar, M. Kaur, G. Kaur, H. Kaur, *Int. Pharm. Sci.* **2011**, *1,* 98-106.

24. D. Xiao, P. M. Davidson, D. H. D’Souza, J. Lin, Q. Zhong, *J. Food Eng*. **2010**, *100*, 194-200.

25. M. Bimakr, R. A. Rahman, F. S. Taip, A. Ganjloo, L. M. Salleh, J. Selamat, A. Hamid, I. S. M. Zaidul, *Food Bioprod. Process*. **2011**, *89*, 67-72.

26. I. Ammar, M. Ennouri, H. Attia, *Ind. Crop. Prod*. **2015**, *64*, 97-104.

27. A. M. Azrie, A. Luqman Chuah, K. Y. Pin, *J. Chem. Pharm. Res*.**2014**, *6,* 172-176.

28. M. H. Ibrahim, H. Z. E. Jaafar, E. Karimi, A. Ghasemzadeh, *Sci. World J*. **2014***, 2014,* 360290.

29. Z. Ismail, H. K. Beh, M. S. R. Hamil, G. Ghafar, M. A. A. Saeed, A. H. Memon, S. Hashim, Publication number: WO2016093692 A1. 16 June 2016.

30. E. Karimi, H. Z. E. Jaafar, S. Ahmad, 2011 *Molecules* **2011**, *16*, 4438-4450.

31. E. Karimi, H. Z. E. Jaafar, A. Ghasemzadeh, M. H. Ibrahim, *Aus. J. Crop Sci*. **2013**, *7*, 1016-1023.

32. E. Karimi, H. Z. E. Jaafar, *Molecules* **2011***, 16,* 6791-6805.

33. R. Gevrenova, N. Denkov, D. Zheleva-Dimitrova, *Pharmacia* **2014**, *61*, 17-23.

34. The Official Hansen Solubility Parameter Site, Http://[www.Hansen-Solubility.com](http://www.hansen-solubility.com) (Accessed: 20 September 2015).

35. C. M. Hansen, *Hansen Solubility Parameters,* in A User's Handbook, 2nd ed. CRC Press, Boca Raton, 2007.

36. J. Gao, Using Hansen solubility parameters (HSPs) to develop antioxidant-packing film to achieve controlled release. Michigan State University, Thesis for Master of Science, 2014.