Asian Journal of Chemistry; Vol. 24, No. 2 (2012), 756-760

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Optimization Extraction of ET-743 from Sea Squirt by Response Surface Methodology and Preparative Chromatographic Separation

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(*Received*: 16 March 2011; *Accepted*: 14 October 2011) AJC-10522

ET-743 is one of the most original anti-tumoral activity compounds from sea squirt. The liquid-liquid extraction is widely used to obtain the target compound directly. Response surface methodology was used to predict the extraction conditions. The preparative chromatography was also applied to purify the target compound from extract. The optimized condition (acetone/methanol (86.67:13.33, v/v), liquid/solid ratio: 8.6 mL/g and 126.0 min dipping time) were estimated using the model equation. 6.47×10^{-4} mg/g of ET-743 can be extracted. By using preparative HPLC column, 5.82×10^{-6} mg of ET-743 was obtained from 1 mL extract. Then, the preparative chromatography with large size of HPLC column was successfully applied to purify the ET-743 from the extract.

Key Words: *Sea squirt*, Anti-cancer compounds, ET-743, Response surface methodology.

INTRODUCTION

In recent years, a new anticancer clinical and preclinical agent has emerged to more effectively investigate the diversified bioactive chemicals by marine life[1]. Many marine invertebrates such as sponges and ascidians exploited as fisheries resources[2] have recently attracted increased attention because of their great economic potential production which can be used in pharmaceutical properties[3].

Ascidiacea (commonly known as the ascidians or *Sea squirts*) is a class in the Urochordata subphylum of sac-like marine invertebrate filter feeders[4,5], which can synthesize a group of molecules called ecteinascidins. One of the most original antitumoral activity compound ET-743 (Fig. 1) was already considered as a promising substance effective against various solid-type tumors[6-10].

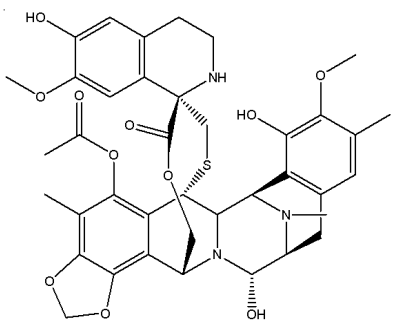


Fig. 1. Molecular structure of ET-743

In order to obtain this compound from *Sea squirt* directly, solvent extraction is the most preferred method. Several conditions should be considered in extraction process. Currently, computer software was frequently used to optimize the extraction condition. Response surface methodology (RSM) with Box-Behnken design (BBD) is one of the significant techniques[11-15]. The main idea of response surface methodology is to use a sequence of designed experiments to obtain an optimal response[16,17] and the experiments will be more easily arranged and interpreted using this efficient design[18,19]. In this study response surface methodology with significant variables was apply to optimize extraction process of ET-743 from *Sea squirt*.

After extraction, the target compound should be purified. Preparative column chromatography with HPLC has been applied for preparation of pure samples[20-22]. In this case, a valid optimization and scale-up method was investigated in this study. The extracted target compound was confirmed by LC-MS and NMR. Compared with previous reports, the present method is more conducive to establish a commercial way.

EXPERIMENTAL

Methanol and acetone were obtained from Duksan Pure Chemical Co., Ltd., (Ansan, Korea). All the other reagents used in the experiment were HPLC or analytical grade. Double distilled water was filtered with a vacuum pump (Division of Millipore, Waters, U.S.A.) and filter (HA - 0.45, Division of Millipore, Waters, U.S.A.) before use. All the samples were

filtered by using a filter (MFS-25, 0.2 μm TF, Whatman, U.S.A.) before injection into the HPLC system.

Chromatographic conditions: Chromatography was performed with a Waters 600s multisolvent delivery system, a Waters 616 liquid chromatography and a Waters 2487 variable wavelength, dual-channel UV detector (Waters Associates, Milford, MA, USA). A syringe with 25 μL injection volume and 5 mL sample loop were used. Data processing was performed with Millennium 3.2 software. Compounds were separated on a 150 mm × 4.6 mm, 5 -μm particle, OptimaPak C_{18} column (RStech, Daejeon, Korea). HPLC separation of ET-743 was conducted by using methanol/water (85/15, v/v) as mobile phase at a flow rate of 0.5 mL/min and the detection was carried out at a wavelength of 210 nm. Micro high speed refrigerated centrifuge (Micro 17R) was from Hanil Science Industrial Co. Ltd., (Korea). LC/MS system (1200 L quadruple) and ^{1}H NMR spectrometer (Inova 400) were purchased from Varian (CA, USA).

Optimum of extraction condition: Optimization of extraction conditions for ET - 743 of *Sea squirt* was extracted by the 15 -run Box-Behnken design method. The independent processing variables were component of extraction solution (X_1), extraction time (X_2) and liquid/solid ratio (X_3). A central composite design was selected for optimization of process variables each at 3 levels with 15 runs including three replicates at the central point. The range and levels of independent variables and code values are presented in Table-1.

TABLE-1
INDEPENDENT VARIABLES THEIR LEVELS
USED FOR BOX-BEHNKEN DESIGN

Independent variables	Level		
	-1	0	1
Component of acetone in methanol (v %)	100	50	0
Liquid/solid ratio (mL/g)	3	6	9
Dipping time (min)	30	90	150

The experimental design was analyzed using the Design-Expert Software (v. 7.1.6, Stat-Ease, Inc., Minneapolis, USA) and fitted to a second-order polynomial regression model containing the coefficient of linear, quadratic and two factors interaction effects. The model equation of response (Y) of the three independent variables (X_1, X_2 and X_3) is:

$$Y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=1+1}^{3} A_{ij} X_i X_j \quad (1)$$

where, Y is the dependent variable, A_0 is the constant coefficient, A_i is the linear coefficient (main effect), A_{ii} is the quadratic coefficient and A_{ij} is the two factors interaction coefficient. Subsequently, three additional experiments were conducted to verify the validity of the statistical experimental strategies.

Samples and columns preparation: The *Sea squirt* was sliced and crushed by a comminuter before extracting. The extraction solutions were separated from insoluble residue by centrifugation (8000 rpm for 20 min) and then precipitated and removed protein by the addition of acetonitrile. After filtration, the extract was collected and stored for injection.

The uniform C_{18} particle (15 μm) purchased from YMC Co. (Kyoto, Japan) was suspended in methanol and degassed by helium. The slurries were pressed into the hollow HPLC column (250 × 4.6 mm) using a pump. After then, the packed column was washed by methanol until a stable baseline was observed.

RESULTS AND DISCUSSION

Determination of ET-743 from extract: Dipping method with different solvents was the most traditional technology for obtaining the ET-743 from *Sea squirt*. Methanol, ethanol, acetone, *n*-hexane and chloroform with different polarity were selected as the extraction solvents. Because of the hydrophilic nature of ET-743, 1.3×10^{-5} and 4.2×10^{-5} mg/g of ET-743 can be obtained by using methanol and acetone. Fig. 2 shows the chromatogram of the extraction by acetone. Each peak was collected and identified by LC/MS and ^{1}H NMR. According to previous research, ET-743 was confirmed. The collected solvent which contained ET-743 was used as standard sample for further experiments.

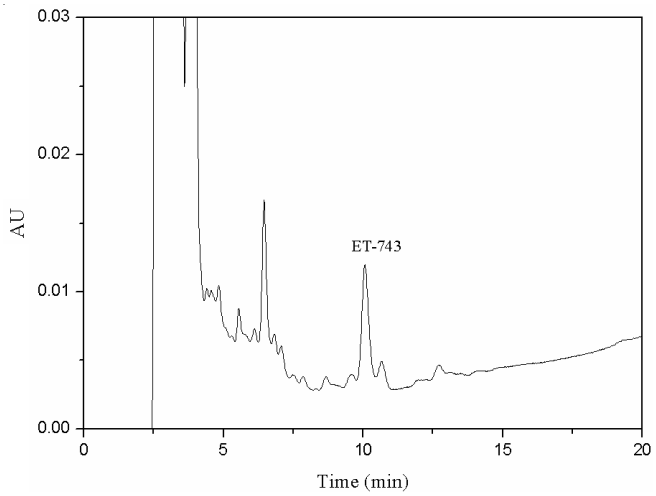


Fig. 2. Chromatogram of acetone extract

Optimize extraction condition by response surface methodology (RSM): Regression models of response: In order to optimize the extraction conditions, a 15-run Box-Behnken design with three variables (extraction solution, solid-liquid ratio and extraction time) and three levels (Tables 1 and 2) were used to fit a second-order response surface and the extraction amount of ET-743 were taken as the response (Table-2). The predicted values of the responses were obtained from quadratic model by fitting the experimental data in eqn. (3).

$$Y = 3.12 - 1.02X_1 + 1.45X_2 + 1.09X_3 - 0.65X_1^2 - 0.32X_2^2 - 1.32X_3^2 - 0.9X_1X_2 - 0.91X_1X_3 + 1.05X_2X_3 \quad (1)$$

where, Y is the predicted response (extraction amount). X_1, X_2 and X_3 are coded values of extraction solution, solid-liquid ratio and extraction time, respectively.

The analysis of ANOVA quadratic regression model is shown in Table-3 and the statistical significance was checked by F-value. The model F-value of 37.54 implies the model is significant with a low probability value (Prob > F = 0.0005). The *p*-value was used to check the significance of each coefficient and also indicated the interaction strength between each independent variable[23]. The smaller the *p*-value, the bigger the significance of the corresponding coefficient[24,25]. In Table-3,

the *p*-value suggests that the model terms should less than 0.05. The precision of a model can be checked by the determination coefficient (R^2). The R^2 implies that the sample variation of 98.54 % for extraction was attributed to the independent variables. In this case, X_1, X_2, X_3, X_1X_2, X_1X_3, X_2X_3, X_1^2 and X_3^2 were significant model terms, respectively.

TABLE-2
BOX-BEHNKEN EXPERIMENTAL DESIGN WITH THE INDEPENDENT VARIABLES

No.	Variables			Response	
				Extraction amount (10^{-4} mg/g)	
	X_1	X_2	X_3	Exp.	Cal.
1	0	1	-1	0.344	0.79
2	-1	0	-1	0.329	0.17
3	1	0	-1	0.134	0.00
4	-1	0	1	3.980	4.17
5	1	0	1	0.138	0.31
6	1	1	0	1.920	1.68
7	-1	-1	0	0.574	0.82
8	0	-1	1	0.511	0.07
9	0	0	0	3.120	3.12
10	-1	1	0	5.790	5.52
11	0	-1	-1	0.070	0.00
12	0	0	0	3.120	3.12
13	0	0	0	3.120	3.12
14	0	1	1	4.990	5.07
15	1	-1	0	0.320	0.58

TABLE-3
ANALYSIS OF VARIANCE OF THE EXPERIMENTAL RESULTS OF THE BOX-BEHNKEN DESIGN

Source	Sum of squares	df	Mean square	F-value	*p*-value Prob > F
Model	53.33	9	5.93	37.54	0.0005
X_1	8.33	1	8.33	52.74	0.0008
X_2	16.73	1	16.73	105.99	0.0001
X_3	9.55	1	9.55	60.52	0.0006
X_1X_2	3.27	1	3.27	20.71	0.0061
X_1X_3	0.33	1	0.33	21.06	0.0059
X_2X_3	4.42	1	4.42	28.00	0.0032
X_1^2	1.57	1	1.57	9.92	0.0254
X_2^2	0.37	1	0.37	2.36	0.185
X_3^2	6.47	1	6.47	40.97	0.0014
Residual	0.79	5	0.16	–	–
Cor total	54012	14	–	–	–

Optimize the extraction condition: In order to optimize the extraction conditions, the selected variables were obtained using the software with the regression equation. The 2D contour plots and 3D response surface were provided as graphical representations of the regression equation. Fig 3 (a) and (b) showed the effect of liquid/solid ratio (mL/g), component of acetone in methanol (v %) and the extraction amount under these conditions when the dipping time was fixed at 1.5 h. The extraction amount of ET-743 increased with the component of acetone increasing until 90 %. And the larger volume of extraction solvent can extract more amount of target compound Fig. 4(a) and (b). In Fig. 5(a) and (b), the largest extraction amount was obtained with the extraction time around 2 h.

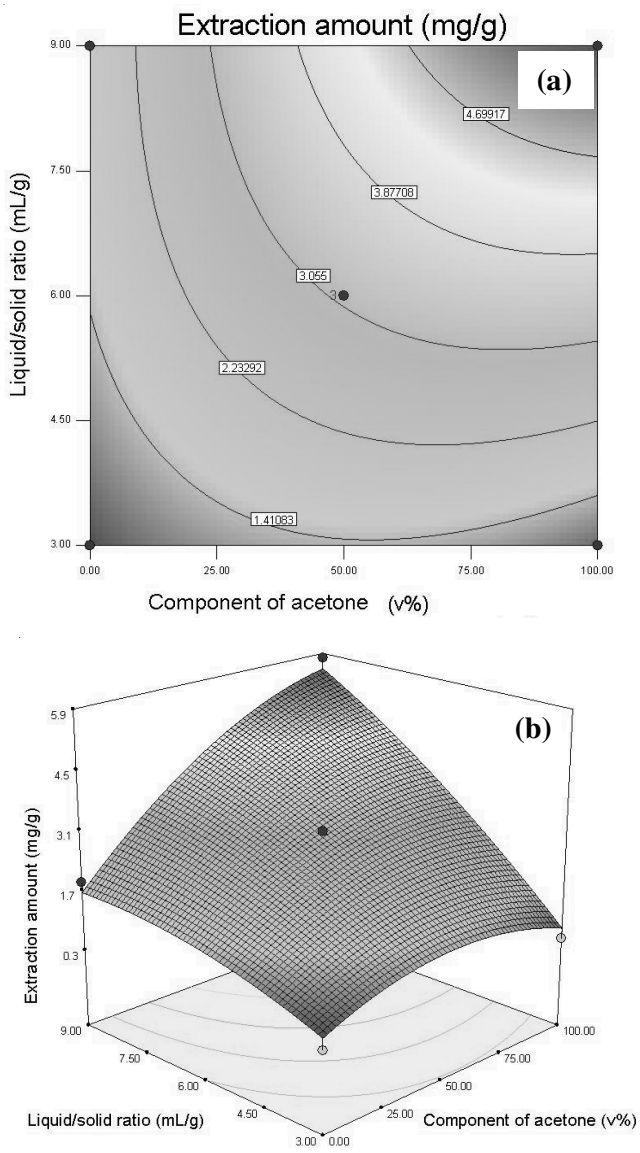
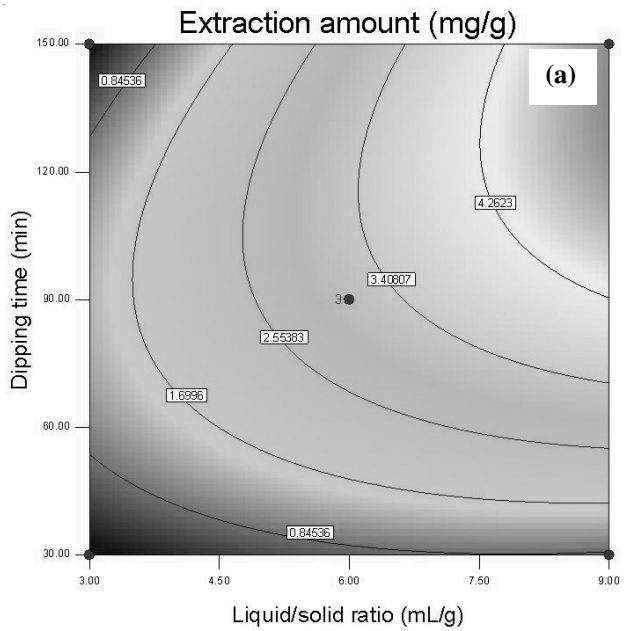


Fig. 3. Effect of component of acetone in methanol, liquid/solid ratio and their reciprocal interaction on extraction amount (with dipping time is constant at 1.5 h) (a: 2D response surface, b: 3D contour plots)



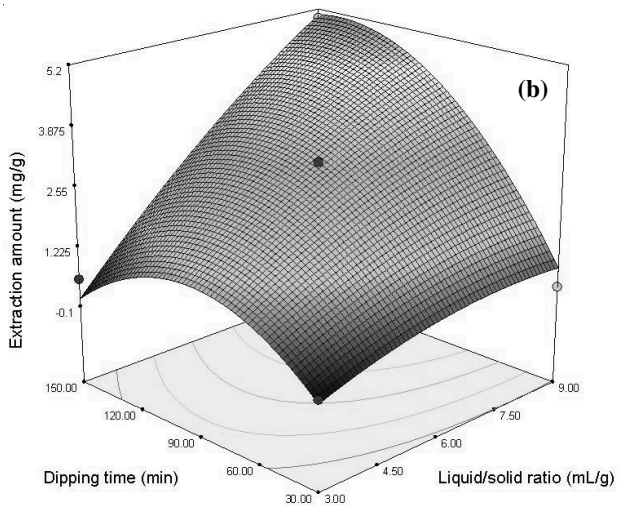


Fig. 4. Effect of component of dipping time, liquid/solid ratio and their reciprocal interaction on extraction amount [with acetone/methanol (50:50, v/v)] (a: 2D response surface, b: 3D contour plots)

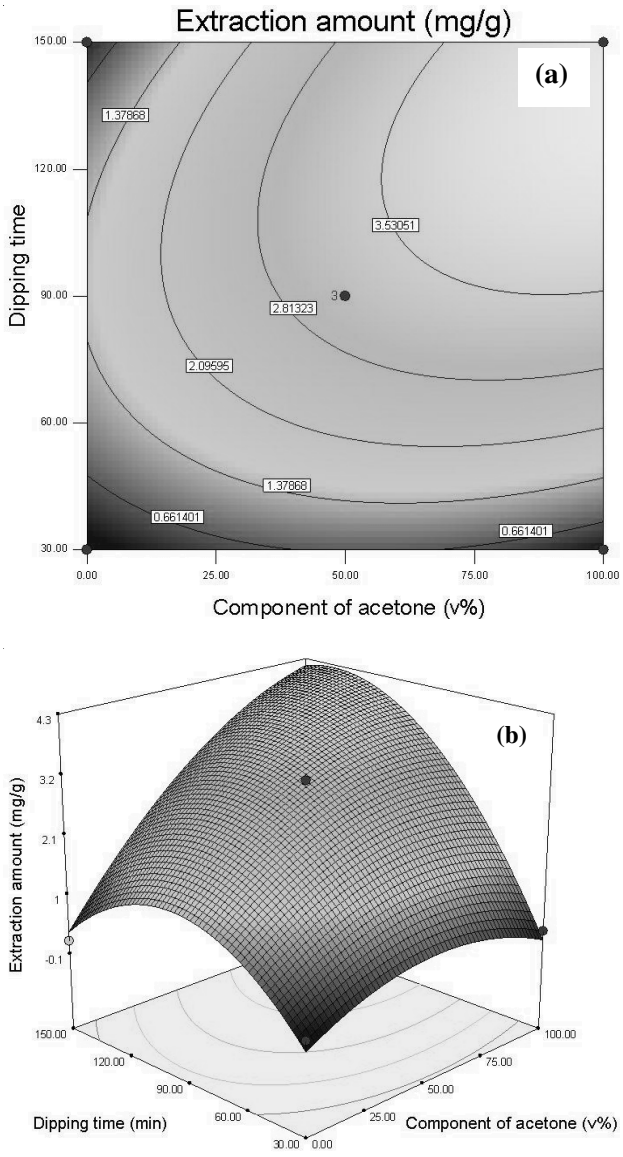


Fig. 5. Effect of component of dipping time, component of acetone in methanol and their reciprocal interaction on extraction amount (with liquid/solid ratio is constant at 6 mL/g) (a: 2D response surface, b: 3D contour plots)

The optimized extraction condition (acetone/methanol (86.67:13.33, v/v), liquid/solid ratio: 8.6 mL/g and 126 min dipping time) for the extraction amount were estimated using Box-Behnken design (Fig. 6). The predicted extraction amount was 6.23×10^{-4} mg/g.

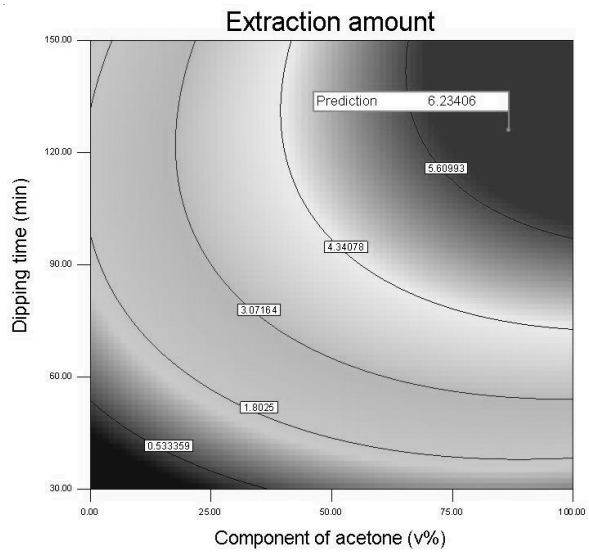


Fig. 6. Predicted optimum values of extraction amount of ET-743 by the Box-Behnken design

Validation of the model: To obtain the largest extraction amount of ET-743, in the optimization process the maximum extraction amount should be set as the purpose. The optimized extraction conditions were applied to extract the ET-743 to verify the prediction from the model. The 6.47×10^{-4} mg/g of extraction amount were confirmed that the response model was adequate for the optimization.

Preparative separation: The ET-743, which was separated by analysis column was collected and used as the standard solution. Larger injection volume (0.1 - 2.0 mL) was used to determine the separation efficiency of preparative column. With the injection volume more than 1 mL, the peaks of target compounds and impurities cannot be separated. Hence, from the results, it was determined that 1 mL injection was suitable for the preparative column. In Fig. 7, 5.82×10^{-6} mg of ET-743 can be obtained in each 1 mL injection of extract.

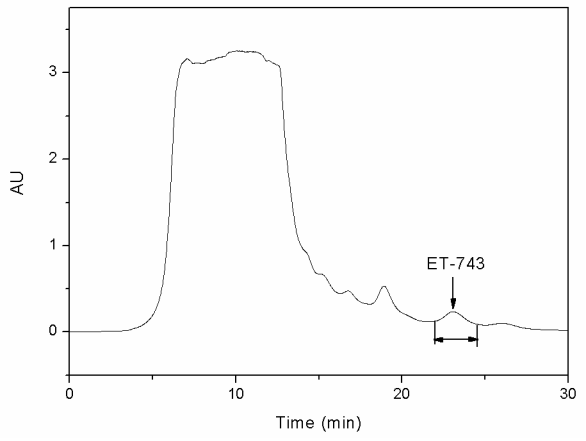


Fig. 7. Chromatogram of extract by using preparative column

Conclusion

In this study, the extraction condition of ET-743 from *Sea squirt* was optimized by using response surface methodology. The coefficient of determination for the model was $R^2 = 98.54$ %. The optimal conditions [acetone/methanol (86.67:13.33, v/v), liquid/solid ratio: 8.6 mL/g and 126.0 min dipping time] were estimated using the model equation. Under the conditions, 6.47×10^{-4} mg/g of ET-743 can be extracted. Then, the preparative chromatography with large size of HPLC column was successfully applied to purify the ET-743 from the extract. Finally, 5.82×10^{-6} mg of ET-743 was obtained from each injection.

ACKNOWLEDGEMENTS

This research was supported by Basic Science Research Program through the National Research Foundation (NRF) of Korea funded by the Ministry of Education, Science and Technology (2011-0002642).

REFERENCES

1. T.L. Simmons, E. Andrianasolo, K. McPhail, P. Flatt and W.H. Gerwick, *Mol. Cancer. Ther.*, **4**, 333 (2005).
2. K.L. Rinehart, *Med. Res. Rev.*, **20**, 1 (2007).
3. J.L. Carballo, S. Naranjo, B. Kukurtzü, F. De La Calle and A. Hernández-Zanuy, *J. World. Aqua. Soc.*, **31**, 481 (2000).
4. R.D. Barnes, Invertebrate Zoology, in Holt-Saunders International, Philadelphia, pp. 1028-1042 (1982).
5. T. Ikuta and H. Saiga, *Developmental Dynamics*, **233**, 382 (2005).
6. E. Stokvis, H. Rosing, L. López-Lázaro and J.H. Beijnen, *J. Mass. Spectrom.*, **39**, 431 (2004).
7. H. Rosing, M.J. Hillebrand, J.M. Jimeno, A. Gómez, P. Floriano, G. Faircloth, R.E. Henrar, J.B. Vermorken, E. Cvitkovic, A. Bult and J.H. Beijnen, *J. Mass. Spectrom.*, **33**, 1134 (1998).
8. A.A. Gab-Alla, *Oceanologia.*, **50**, 239 (2008).
9. S. Marchini, E. Marrazzo, R. Bonomi, G. Chiorino, M. Zaffaroni, L. Weissbach, F. J. Hornicek, M. Broggini, G.T. Faircloth and M. D'lncalci, *Eur. J. Cancer*, **41**, 323 (2005).
10. M. Zewail-Foote and L.H. Hurley, *Eur. J. Med. Chem.*, **42**, 2493 (1999).
11. J.E. Cacace and G. Mazza, *J. Food. Sci.*, **68**, 240 (2003).
12. K. Zhong and Q. Wang, *Carbohydr. Polym.*, **80**, 19 (2010).
13. C. Liyana-Pathirana and F. Shahidi, *Food. Chem.*, **93**, 47 (2005).
14. T. Zhu, H.J. Heo and K.H. Row, *Carbohydr. Polym.*, **82**, 106 (2010).
15. E. Karacabey and G. Mazza, *Food. Chem.*, **119**, 343 (2010).
16. Y. Sun, T. Li, J. Yan and J. Liu, *Carbohydr. Polym.*, **80**, 242 (2010).
17. D.V. Cortez and I.C. Roberto, *J. Chem. Technol. Biotechnol.*, **85**, 33 (2010).
18. Y. Wu, S.W. Cui, J. Tang and X. Gu, *Food. Chem.*, **105**, 1599 (2007).
19. G. Yin and Y. Dang, *Carbohydr. Polym.*, **74**, 603 (2008).
20. D. Han, M. Tian and K.H. Row, *J. Liquid Chromatogr. Rel. Technol.*, **32**, 2407 (2009).
21. D.H. Kang and K.H. Row, *J. Chromatogr. A*, **949**, 217 (2002).
22. I.J. Kim, S.B. Hong and K.H. Row, *J. Chromatogr. A*, **949**, 275 (2002).
23. H. Xu, L.P. Sun, Y.Z. Shi, Y.H. Wu, B. Zhang and D.Q. Zhao, *Biochem. Eng. J.*, **39**, 66 (2008).
24. J.Z. Liu, L.P. Weng, Q.L. Zhang, H. Xu and L.N. Ji, *World J. Microbiol. Biotechnol*., **19**, 317 (2003).
25. Y. Li, F. Cui, Z. Liu, Y. Xu and H. Zhao, *Enzyme Microb. Technol.*, **40**, 1381 (2007).