PRODUCTION OF A DIACYLGLYCEROL-ENRICHED SAFFLOWER OIL USING LIPASE-CATALYZED GLYCEROLYSIS: OPTIMIZATION BY RESPONSE SURFACE METHODOLOGY

Sayed Mohammad Sahafi[1]*, Sayed Amir Hossein Goli[1] and Mahdi Kadivar[1]

Isfahan University of Technology, Iran

Abstract

Purpose: This study aimed to develop a model for producing diacylglycerols (DAGs) in safflower oil. Oils with a high diacylglycerol (1,3-DAG) content have attracted considerable attention due to their favourable effects in preventing many diseases. There are valid scientific reports on the effects of diacylglycerol oil in preventing the accumulation of body fat and obesity, increased sensitivity of cells to insulin, reduced sodium concentration in the blood, LDL and cholesterol and blood pressure in people with atherosclerotic disease.

Design/methodology/approach: In this study, 1,3-DAG was synthesized from safflower oil using the glycerolysis reaction in a solvent-free system with lipozyme TL IM as a biocatalyst. A D-optimal design was used to model and optimize the reaction conditions. Evaluation of the resulting model enabled the determination of optimal reaction conditions for glycerolysis, aiming at a high DAG yield. The glycerolysis reaction was optimized with four factors of temperature, time, molar ratio of glycerol to oil and enzyme percentage.

Findings: The DAG content of the product was dependent on all parameters examined except reaction temperature. DAG formation increased with in-

International Journal of Food, Nutrition and Public Health (IJFNPH)
Vol. 5 No. 4, 2012

[1] Department of Food Science, College of Agriculture, Isfahan University of Technology, Isfahan, IRAN 84156-83111
* E-mail address: sm.sahafi@yahoo.com

creasing substrate ratio and decreasing enzyme load and reaction time. The highest DAG production was 52% (w/w, on the basis of total fat) and optimal conditions were found to be 0.75% enzyme, 5.3 g glycerol, a temperature of 46.9 °C and a reaction time of 4 h. In these conditions, after purification, DAG content increased to 53.84. The content of sn-1,3-DAG was higher than that of sn-1,2-DAG (70:30) under all reaction conditions.

Keywords: Safflower oil, Lipozyme TL IM, Glycerolysis, Diacylglycerol oil

Paper type: Research paper

INTRODUCTION

Fats and oils are essential ingredients and important components in our daily diet (Lo *et al.*, 2008). They are important sources of energy, essential fatty acids and fat-soluble vitamins, and impart excellent flavour, texture and palatability to the food (Wang *et al.*, 2009). However, studies have documented the health concerns associated with diets high in fats. A high intake of fats has been responsible for the high incidence of cardiovascular disease, hypertension, and obesity. Diacylglycerols (DAGs), are esters of glycerol with two fatty acids (FAs) which have two structural isomers, i.e., sn-1,2(2,3) DAG and sn-1,3 DAG. DAGs are the most widely used emulsifiers in food and pharmaceutical industries. These new healthy oils are naturally present as minor components in various edible oils and fats (Yang *et al.*, 2004), and are digested and metabolized in a different way, which significantly affects the body weight increase (Kristensen *et al.*, 2005a).

Recently, it has been reported that the use of DAG as a cooking oil has health benefits (Lo *et al.*, 2004). In early 1999, Kao introduced a novel application of diacylglycerol oil as a functional cooking oil to Japanese consumers. Researchers at Kao have found that DAG oil has metabolic characteristics that are different from triacylglycerol (TAG) oils (Lo *et al.*, 2008). DAG oil (which is 1,3-DAG) is the first and only oil clinically shown to be stored less as fat in the body compared to other edible oils. The energy value of DAG oil and TAG oil (with similar fatty acid composition) was virtually identical, that is 9.30 and 9.46 kcal/g, respectively. Moreover, no significant differences in absorption rates (96.3% in both cases) were observed. Therefore the beneficial characteristic of DAG oil is not due to a difference in energy value or absorption rate, but is attributable to the different metabolic mechanism

of TAG and 1,2-DAG compared to 1,3-DAG after absorption by intestinal epithelial cells (Blasi *et al.*, 2007).

Consumption of DAG oils could have several positive effects on the human body, including increased oxygen consumption, energy consumption, beta-oxidation in the liver and small intestine to produce energy, blood insulin and sensitivity of cells to insulin, blood fibrinogen concentration and reduction in the amount of platelets, activity of lactate dehydrogenase, sodium concentration in the blood, weight and body fat and amount of blood glucose (Matsuo, 2004; Morita and Soni, 2009).

DAGs can be produced chemically or enzymatically through esterification (glycerol and FA), glycerolysis (glycerol and TAG) and partial hydrolysis (TAG and water) processes in organic solvents and in a solvent-free system (Weber and Mukherjee, 2004). The enzymatic processes are ideal because of their mild reaction conditions (temperature and pressure), energy conservation, availability from a wide range of sources, potential for improvement via genetic engineering, higher product purity and quality, the regioselectivity (nonspecific and 1,3-specific) and enantioselectivity of lipases, the possibility of recoverability and recyclability, less environmental impact and the safety of the end products (Guo and Sun, 2007). Among these enzymatic approaches for DAG preparation, glycerolysis of TAG in a solvent-free system has been proven to be efficient for the preparation of sn-1,3-DAG (Yeoh *et al.*, 2009). Response surface methodology (RSM) is a set of mathematical and statistical methods developed for modelling and to find alone or in combination with a number of experimental factors that lead to optimum responses. It also offers a large amount of information from a small number of experiments (Ferreira-Dias *et al.*, 2001; Kristensen *et al.*, 2005b).

In this study, DAG is produced through lipase-catalyzed glycerolysis of safflower oil using immobilized *Thermomyces lanuginosus* lipases (TL IM), in a solvent-free system. As there are many factors affecting DAG production, the objective of this study was to optimize the reaction time, enzyme load, reaction temperature and substrate molar ratio (safflower oil/glycerol) by RSM for maximum yield of diacylglycerol.

MATERIALS AND METHODS

Safflower seeds were provided by the Oil Crop Development Centre in Isfahan, Iran. All solvents and chemicals used were of analytical grade

and obtained from Merck (Darmstadt, Germany). Silylation reagents (TMCS (trimethylchlorosilane) and BSTFA (N,O-bis[trimethylsilyl] trifluoroacetamide), fatty acid (palmitic, stearic, oleic, linoleic and linolenic acids), mono and diacylglycerol (1-linoleoyl-rac-glycerol and 1,3-dilinoleoyl-rac-glycerol) as standard for GC analysis were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). lipozyme TL IM (*Thermomyces lanuginosus* lipase immobilized) was generously supplied by Novozymes A/S (Tehran, Iran).

SOLVENT EXTRACTION

Hexane was used to extract oil from the safflower seeds. The milled seeds were exposed to hexane at ratio of 1:2 at room temperature for 12 h, twice. The extract was filtered through filter paper for removal of particles. Finally, hexane was removed by rotary vacuum evaporator.

ANALYSIS OF ACYLGLYCEROLS

GLYCEROLYSIS REACTION

The reaction mixture consisted of safflower oil (30 g) and glycerol in different molar ratios (glycerol/oil) and immobilized lipase (the amount of lipase was based on the weight of oil). Reactions were carried out under varied conditions of reaction time and reaction temperature. The reaction was stirred using an orbital shaker at 400 rpm. Samples of the reaction mixture were filtrated through 0.45 µl to remove the enzyme lipases before analysis. The molar ratio of glycerol/oil, enzyme load, reaction time and reaction temperature used varied according to the experimental design followed.

SAMPLE SOLUTION

The procedure involves conversion of the mono and diglycerides into more volatile trimethylsilyl ether derivatives using BSTFA and TMCS in pyridine. Prior to analysis, the reaction products were filtered through a 0.45 mm nylon membrane filter to separate the lipase particles. 50 mg of oil was transferred into a 2.5 ml screw cap vial with Teflon faced septa and 50 µl pyridine was added. Pyridine, in addition to the role of solvent, acts as an electron receptor. The silylating agents were then added, including 0.2 µl BSTFA and 0.1

μl TMCS. The vial was closed and shaken vigorously. The reaction mixture was then heated in a heating device at 70 °C for 20 min. It should be noted that in this reaction, humidity is strictly excluded and should not enter the vial. The reaction was carried out twice and duplicate injections were made per reaction.

DETECTION OF ACYLGLYCEROLS BY GLC

The silylated sample (2 μl) was analyzed using an Agilent 6890N gas chromatograph equipped with split-splitless injector and with a flame ionization detector. The column used was a HP-5 (30 m, 0.32 mm i.d., 0.25 μm film thickness) column. The carrier gas was helium (ultra high purity) with a flow rate of 5 mL/min. The injector and detector temperatures were 320 and 350 °C. The oven temperature was programmed from 80 to 320°C at a rate of 10°C/min and held for 26 min. The injector was used in split mode with a ratio of 1:30.

EXPERIMENTAL DESIGN

An appropriate range for each independent variable was determined for RSM. A statistical method—D-optimal design—was adopted to optimize the reaction conditions for maximum production of DAG analog from the enzymatic reaction. Enzyme load X_1 (%), reaction time X_2 (h), reaction temperature X_3 (°C) and mole ratio of glycerol to safflower oil X_4 were used as the variables to maximize the response (yield of DAG analog). The experimental design included 25 experiments of four variables at three levels (−1, 0, +1). Table 1 gives the range of variables employed. The experimental plan was designed and the results obtained were analyzed using Design Expert version 7.1.6 (Stat-Ease Inc., Minneapolis, MN) software to build and evaluate models.

Table 1. Experimental range and values of the independent process variables in the RSM

Independent variables	Symbols	Range and levels		
		-1	0	+1
Enzyme load (%)	X_1	5	10	15
Reaction time (h)	X_2	8	16	24
Reaction temperature (ºC)	X_3	40	50	60
Mole ratio of Gly/oil	X_4	1:1	2:1	3:1

RESULTS AND DISCUSSION

MODEL FITTING

The actual set of experiments performed (experimental runs 1–25) and the yield of diacylglycerol obtained are shown in Table 2. A second-order polynomial equation was used to express the DAG yield oil (Y) as a function of the independent variables, as shown below:

$$Y = 31.50697 - 1.07083\,X_1 - 1.10035\,X_2 + 2.68184\,X_4 + 0.020458\,X_2^2 - 0.26886\,X_4^2 - 0.024282\,X_1X_2 + 0.11245\,X_1X_4 + 0.038806\,X_2X_4 \quad \text{Equation 1}$$

Exp. No.	Variable levels				Yield (%)		Residual
	X1	X2	X3	X4	Experimental	Predicted	
1	5	8	60	1:1	35.55	35.49	0.06
2	5	24	50	1:1	32.16	31.91	0.25
3	5	8	60	2:1	38.74	38.64	0.1
4	10	8	60	3:1	23.36	24.21	-0.85
5	15	8	40	1:1	41.37	42.02	-0.65
6	15	8	60	1:1	40.35	40.07	0.28
7	15	8	40	3:1	34.96	34.85	0.11
8	15	24	40	3:1	33.19	32.88	0.31
9	10	16	50	2:1	42.18	41.27	0.91
10	10	16	50	2:1	31.48	32.40	-0.92
11	5	8	50	3:1	42.13	41.85	0.28
12	15	16	60	3:1	39.71	39.58	0.13
13	10	24	60	1:1	44.15	43.79	0.36
14	5	24	60	3:1	39.36	39.37	-0.01
15	5	24	40	1:1	38.11	38.76	-0.65
16	15	8	60	1:1	33.76	32.63	1.13
17	15	24	40	3:1	41.63	41.92	-0.29
18	15	8	40	3:1	38.77	38.73	0.04
19	5	24	60	3:1	38.05	37.48	0.57
20	5	16	60	1:1	41.55	41.66	-0.11
21	5	24	40	2:1	39.80	41.27	-1.47
22	5	16	40	3:1	39.03	38.76	0.27
23	5	8	40	1:1	35.21	34.85	0.36

Table 2. Experimental settings of the factors and responses of dependent variables for the reaction by RSM

24	15	24	60	2:1	32.86	32.40	0.46
25	15	24	40	1:1	38.72	39.37	-0.65

The accuracy of the models was evaluated by coefficient of determination R^2 and adjusted R^2 values (0.9831 and 0.9595, respectively) and the model of quadratic polynomial was suggested by the software.

EFFECT OF THE PARAMETERS ON DAG PRODUCTION

ENZYME LOADING (%)

As shown in Equation 1, the amount of enzyme produces a linear effect on DAG yield and interacts with time and glycerol content. Response surfaces can be illustrated by presenting the response as a function of one factor and keeping the other constant. Therefore, by increasing the enzyme load from 5 to 15 per cent, DAG content decreased; in other words, there was a negative linear effect on DAG production. Similar results have been observed by Kruger *et al.* (2010), who explained that by increasing the enzyme load, the efficiency of DAG production increased significantly.

According to Equation 1, there is an interaction between enzyme load, time and glycerol content. To visualize the combined effects of the two factors on the response, the response surface and contour plots were generated for each of the fitted models in functions of two variables, while keeping the other two variables at the central values. Fig. 1 denotes the response surface and contour plots of the DAG yield as a function of enzyme load/time and enzyme load/glycerol content at a temperature of 50°C.

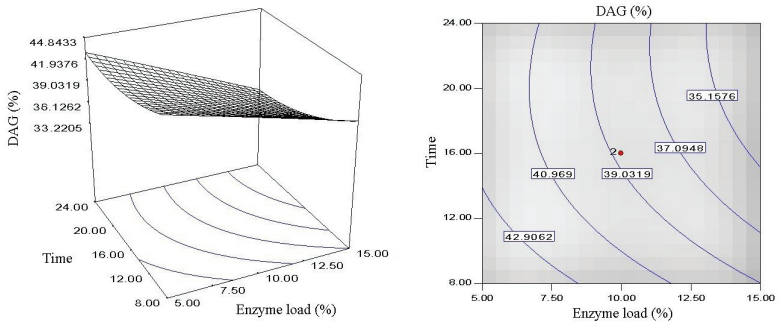

Fig. 1. Response surface and contour plots of the DAG yield as a function of time and enzyme load (%) at centre levels of temperature and glycerol content

As can be seen in Fig. 1, by increasing enzyme load, the content of DAG decreased significantly, while the effect of time was minor in this case. The contour plot shows that maximum DAG content was obtained by using 5 to 8.5% enzyme and 8 to 14 h reaction time.

Fig. 2 shows response surface and contour plots regarding the effect of enzyme load and glycerol content on the DAG yield at 50°C with a reaction time of 16 h. There was significant interaction between enzyme load and glycerol content. Based on the response surface plot, as the amount of glycerol increased, the DAG content first increased and then declined with a gentle slope. The contour plot showed that maximum DAG content was obtained by using 5 to 7% enzyme and 4 to 9 g glycerol. Ferreira-Dias *et al.* (2001) also evaluated the response of glycerol and enzyme load for producing DAG. They found that by increasing the glycerol, the DAG content was enhanced, but in contrast, the DAG content decreased when the enzyme load increased.

TIME

According to Equation 1, time showed a negative quadratic effect on DAG yield and interacted with glycerol content. By increasing reaction time from 8 to 24 h, DAG content decreased**.** In fact, with increasing reaction time, the enzyme starts to use the DAGs as a substrate in order to convert them to MAG and TAG. As shown in Equation 1, there is an interaction between reaction time and amount of glycerol. Fig. 3 shows response surface and contour plots regarding the effect of reaction time and glycerol content on the DAG yield at 50°C with an enzyme load of 10%. As mentioned before, increased

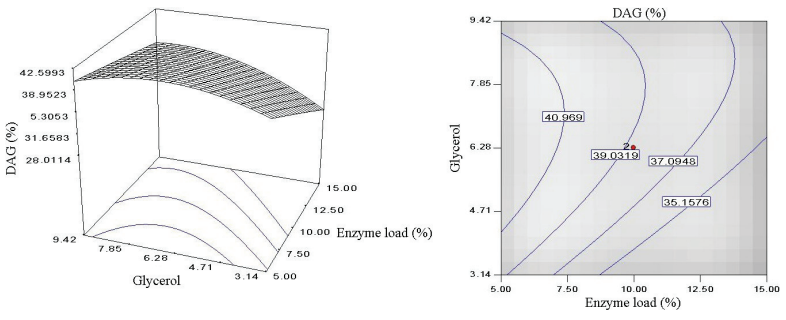

Fig. 2. Response surface and contour plots of the DAG yield as a function of enzyme load (%) and glycerol content at centre levels of temperature and time

reaction time had a slight effect on DAG production, while increased glycerol content resulted in increased DAG yield. The contour plot shows that the maximum DAG yield will be obtained by 8-10 h reaction time and 5-9 g glycerol.

GLYCEROL

As shown in Equation 1, the amount of glycerol (g) produced a quadratic effect on DAG yield. In order to study the effect of glycerol molar ratio on DAG content, the other factors, including time, temperature and enzyme load, were kept constant. It should be noted that the glycerol had no interaction with any other factors. By increasing the glycerol up to 7.8 g, the DAG yield increased significantly, but more glycerol (> 7.8) had a negative effect on DAG yield and caused it to decline slightly. Noureddini and Harmeier (1998) also studied the effect of glycerol on DAG yield and found that by increasing glycerol, the DAG yield increased and reached a constant point.

OPTIMIZATION OF PROCESS

At the optimum point, the maximum predicted value of the DAG yield was 48.44%, which is achieved at 46.9 °C temperature, 4 h time, 0.75% enzyme load (weight of oil) and a molar ratio of glycerol to oil of 2:1. The adequacy of the predictive model was examined by performing independent experiments at the optimal conditions. Verification results revealed that the predicted values were reasonably close to observed values, which are 52.08 and 52 respectively. After removal of impure materials such as unreacted glycerol and fatty acid via the alkaline method, DAG content increased to 53.84%.

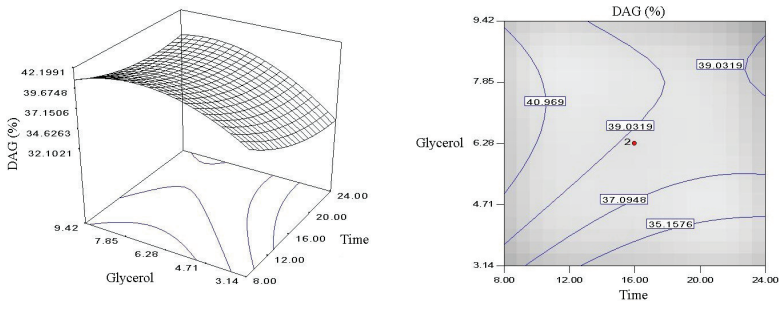

Fig. 3. Response surface and contour plots of the DAG yield as a function of reaction time (h) and glycerol content (g) at centre levels of temperature and enzyme load

CONCLUSION

In conclusion, for the first time, a model for producing DAG in safflower oil with RSM has been successfully developed and glycerolysis has been optimized to produce DAG. Due to the notable advantages of DAG when compared with conventional oil, and the merit of enzyme reaction rather than chemical reaction, this oil can be scaled up in a pilot plan, or even in factories.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Isfahan University of Technology for financial support, B. Bahrami for his technical assistance and M. Jafari and B. Rashidi for their compassionate support.

REFERENCES

Blasi, F., Cossignani, L., Simonetti, M.S. and Damiani, P. (2007), "Biocatalysed synthesis of sn-1,3-diacylglycerol oil from extra virgin olive oil", *Enzyme and Microbial Technology,* Vol. 41, pp. 727-732.

Ferreira-Dias, S., Correia, A.C., Baptista, F.O. and da Fonseca, M.M.R. (2001), "Contribution of response surface design to the development of glycerolysis systems catalyzed by commercial immobilized lipases", *Journal of Molecular Catalysis B: Enzymatic,* Vol. 11, pp. 699-711.

Guo, Z. and Sun, Y. (2007), "Solvent-free production of 1,3-diglyceride of CLA: strategy consideration and protocol design", *Food Chemistry,* Vol. 100, pp. 1076-1084.

Kristensen, J.B., Xu, X., and Mu, H. (2005a), "Diacylglycerol synthesis by enzymatic glycerolysis: screening of commercially available lipases", *Journal of the American Oil Chemists' Society,* Vol. 82 No. 5, pp. 329-334.

Kristensen, J.B., Xu, X. and Mu, H. (2005b), "Process optimization using response surface design and pilot plant production of dietary diacylglycerols by lipase-catalyzed glycerolysis", *Journal of Agricultural and Food Chemistry,* Vol. 53, pp. 7059-7066.

Kruger, R.L., Valerio, A., Balen, M., Ninow, J.L., Oliveira, J.V., Oliveira, D.D. and Corazza, M.L. (2010), "Improvement of mono and diacylglycerol production via enzymatic glycerolysis in tert-butanol system", *European Journal of Lipid Science and Technology,* Vol. 112, pp. 921-927.

Lo, S.K., Baharin, B.S., Tan, C.P. and Lai, O.M. (2004), "Enzyme-catalyzed production and chemical composition of diacylglycerols from corn oil deodorizer distillate", *Food Biotechnology*, Vol. 18 No. 3, pp. 265-278.

Lo, S.K., Tan, C.P., Mohd, K.L., Yusoff, S.A. and Lai, O.M. (2008), "Diacylglycerol oil-properties, processes and products: a review", *Food Bioprocess Technology*, Vol. 1, pp. 223-233.

Matsuo, N. (2004), "Nutritional characteristics and health benefits of diacylglycerol in foods", *Food Science and Technology Research*, Vol. 10 No. 2, pp. 103-110.

Morita, O. and Soni, M.G. (2009), "Safety assessment of diacylglycerol oil as an edible oil: a review of the published literature", *Food and Chemical Toxicology*, Vol. 47, pp. 9-21.

Noureddini, H. and Harmeier, S.E. (1998), "Enzymatic glycerolysis of soybean oil", *Journal of the American Oil Chemists' Society*, Vol. 75 No. 10, pp. 1359-1365.

Wang, Y., Zhao, M., Ou, S., Xie, L. and Tang, S. (2009), "Preparation of a diacylglycerol-enriched soybean oil by phosphalipase A1 catalyzed hydrolysis", *Journal of Molecular Catalysis B: Enzymatic*, Vol. 56, pp. 165-172.

Weber, N. and Mukherjee, K.D. (2004), "Solvent-free lipase-catalyzed preparation of diacylglycerols", *Journal of Agricultural and Food Chemistry*, Vol. 52, pp. 5347-5353.

Yang, T., Zhang, H., Mu, H., Sinclair, A.J. and Xu, X. (2004), "Diacylglycerols from butterfat: production by glycerolysis and short-path distillation and analysis of physical properties", *Journal of the American Oil Chemists' Society*, Vol. 81 No. 10, pp. 979-987.

Yeoh, C.M., Choong, T.S.Y., Abdullah, L.C., Yunus, R. and Siew, W.L. (2009), "Influence of silica gel in production of diacylglycerol via enzymatic glycerolysis of palm olein", *European Journal of Lipid Science and Technology*, Vol. 111, pp. 599-606.

ABOUT THE AUTHORS

Sayed Mohammad Sahafi graduated with a BSc degree in 2007 and an MSc degree in 2011 from the Department of Food Science and Technology, Isfahan University of Technology, Iran. His thesis focused on "Process

optimization using response surface methodology to produce diacylglycerol oil from safflower oil by enzymatic glycerolysis". Since graduating, he has worked on several scientific projects, including "Biodiesel Production from Waste Oils" and "Novel Oilseed of *Dracocephalum kotschyi*".

Dr Sayed Amir Hossein Goli obtained his PhD degree in food science from Isfahan University of Technology, Iran, in 2009, with a thesis entitled "Chemical production of conjugated linoleic acid (CLA) and its use in margarine formulation". His teaching experience is in Advanced Oil Technology (MSc), Oil Technology (BSc), Principles of Food Processing (BSc), Principles of Fishery Products Processing (BSc) and Beverage Technology (BSc). He has published over ten papers in ISI journals and thirty conference papers. He has supervised more than ten MSc students and published one book on "Edible Oil Processing" in Persian.

Professor Mahdi Kadivar obtained his PhD degree in food science from the University of Saskatchewan, Canada, in 2001, with a thesis entitled "Studies on integrated processes for the recovery of mucilage, hull, oil and protein from Solin®". Presently, he is head of the main library at Isfahan University of Technology. He is a member of the Institute of Food Technologists (IFT), the American Association of Cereal Chemists (AACC) and American Oil Chemists Society (AOCS). His teaching experience includes Cereal Science and Technology (BSc), Advanced Cereal Science (MSc), Principles of Food Packaging (BSc), Quality Control in Food Science (BSc) and Advanced Food Packaging (PhD). He has published approximately fifty ISI papers and thirty-five conference articles. He has owned three patents, and has supervised fifty-four Master and seven PhD students to completion. He has also authored four books and carried out four research projects for executive bodies in Iran.