APPENDICES

APPENDIX A

Chemical preparation

1. Acetic acid solution

One percent acetic acid was prepared as followed: acetic acid 10 ml dissolve in 100 ml distilled water and made up to 1000 ml in a volumetric flask

2. Phosphate buffer (PBS) solution

Phosphate buffer at pH 7.4 was prepared according to Perrin and Dempsey (1974).

1. Prepared solution A (0.05 M dibasic sodium phosphate) as followed: dibasic sodium phosphate (Na_2HPO_4) 7.8 g was dissolved in 1000 ml distilled water

2. Prepared solution B (0.05 M monobasic sodium phosphate) as followed: monobasic sodium phosphate (NaH_2PO_4) 8.90 g was dissolved in 1000 ml distilled water

3. Mixed 40.5 ml solution A and 9.5 ml solution B together and made up to 1 L in a volumetric flask

3. The ABTS solution (7 mM ABTS)

Seven mM ABTS was prepared by dissolving0.0192 g ABTS [2,29-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt)] in 5 ml distilled water

4. Potassium persulfate solution: (140 mM $K_2S_2O_8$)

One hundred forty mM $K_2S_2O_8$ was prepared by dissolving 0.3784 g di-potassium peroxodisulfate in 10 ml distilled water

APPENDIX B

Determination of carotenoids

1. Standard curve of carotenoids in ethanol

The standard curve of carotenoids was prepared using the standard β-carotene. The method has been described in the AOAC (2002) as following detail:

1. The 0.0100 g standard β-carotene was dissolved in 2.5 ml ethanol and made up to 100 ml in a volumetric flask

2. Pipetted 10 ml of solution obtained from step 1and made up to 50 ml with ethanol

3. Pipetted 1, 2, 3, 4, 5, 6 and 7 ml of solution obtained from step 2 and made up each volume to 25 ml in a volumetric flask with ethanol

4. Measured the absorbance of each sample dilution using spectrophotometer at 450 nm

5. The standard curve was plotted between the concentration of β-carotene (ppm) and the corresponded absorbance as shown in figure A1

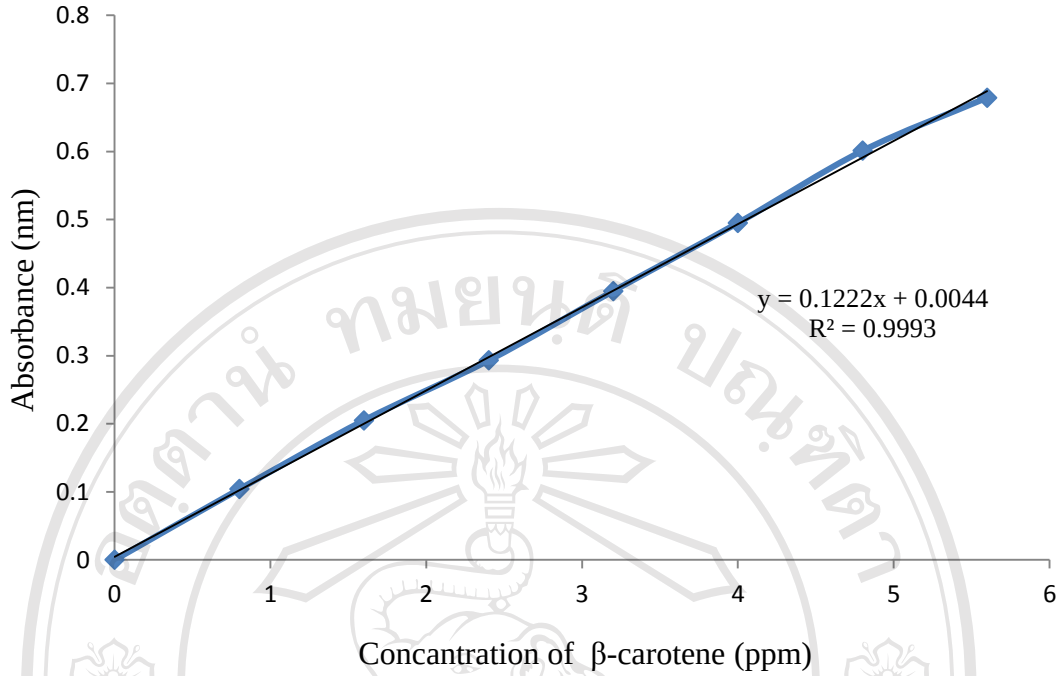


Figure A1 Standard curve of β-carotene in ethanol

2. Standard curve of carotenoids inpH 7.4 phosphate buffer solution

The standard curve of carotenoids was prepared using the standard β-carotene as described in the AOAC method (2002) as followed:

1. The 0.0100 g standard β-carotene was dissolved in 2.5 ml PBS and made up to 100 ml in a volumetric flask

2. Pipetted 10 ml of solution obtained from step 1and made up to 50 ml in a volumetric flask with PBS

3. Pipetted 1, 2, 3, 4, 5, 6 and 7 ml of solution obtained from step 2 and made up each volume to 100 ml in a volumetric flask with PBS

4. Measured the absorbance of each sample dilution using spectrophotometer at 450 nm

5. The standard curve was plotted between the concentration of β-carotene (ppm) and the corresponded absorbance as shown in figure A2

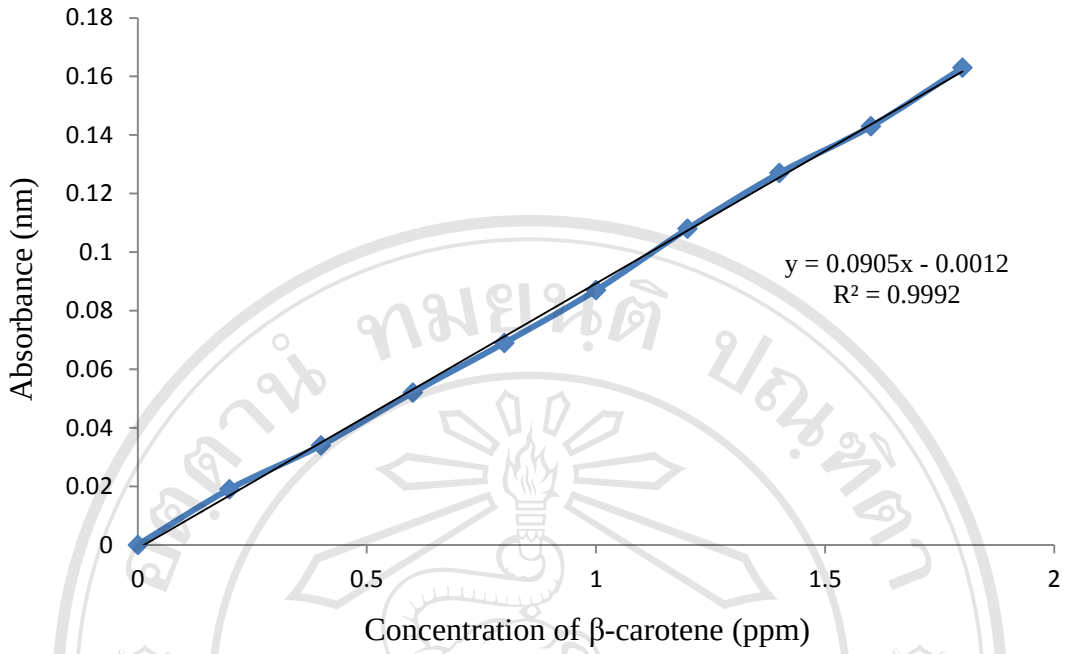


Figure A2 Standard curve of β-carotene in PBS

3. Determination of total carotenoids

Determination of the total carotenoids was carried out according to Ribeiro *et al* (2008) with slight modification. Firstly, 0.1 g of sample was dissolved in 100 ml of ethanol or PBS and measured the absorbance at 450 nm. Then, calculated the quantities of total carotenoids using the linear equations obtained from the standard curves of carotenoids in ethanol (Figure A1) or PBS (Figure A2), respectively.

For example, if the absorbance of sample A was 0.495, the quantity of total carotenoids (x) would calculate as following:

Linear equations was $y = 0.1222x + 0.0044$

$0.495 = 0.1222x + 0.0044$

$0.495 - 0.0044 = 0.1222x$

$0.4906 = 0.1222x$

$x = 0.4906/0.1222$

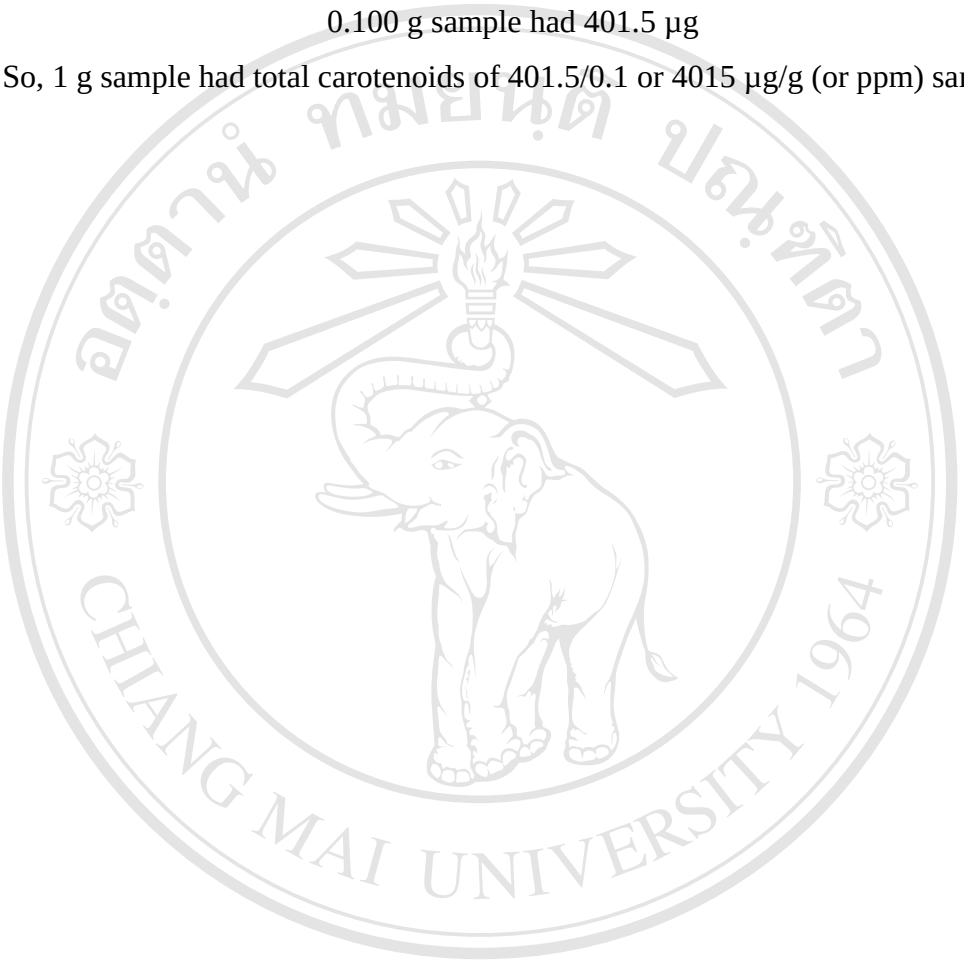
$x = 4.015$ µg/ml (or ppm)

1 ml ethanol had total carotenoids of 4.015 µg

100 ml ethanol had 4.015x100 = 401.5 µg

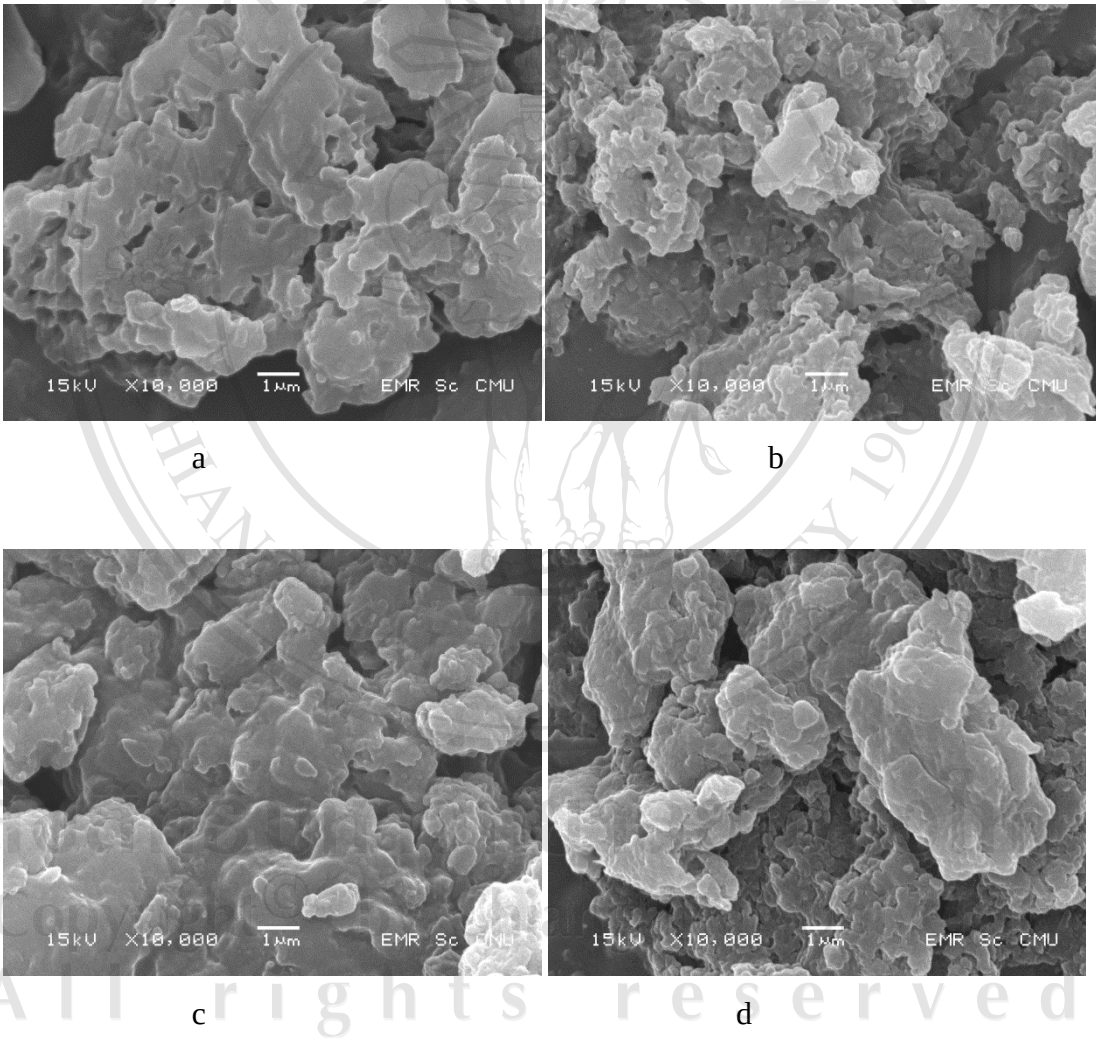
0.100 g sample had 401.5 µg

So, 1 g sample had total carotenoids of 401.5/0.1 or 4015 µg/g (or ppm) sample



APPENDIX C

Physical characteristics of carotenoids encapsulated chitosan-TPP Determined using Scanning Electron Microscopy (SEM)



a b

c d

Figure A3SEM image of 2.0% (w/v) carotenoids encapsulated chitosan
(a) without TPP (b) with 0.5% (w/v) TPP
(c) with 1.0% (w/v) TPP (d) with 2.0% (w/v) TPP

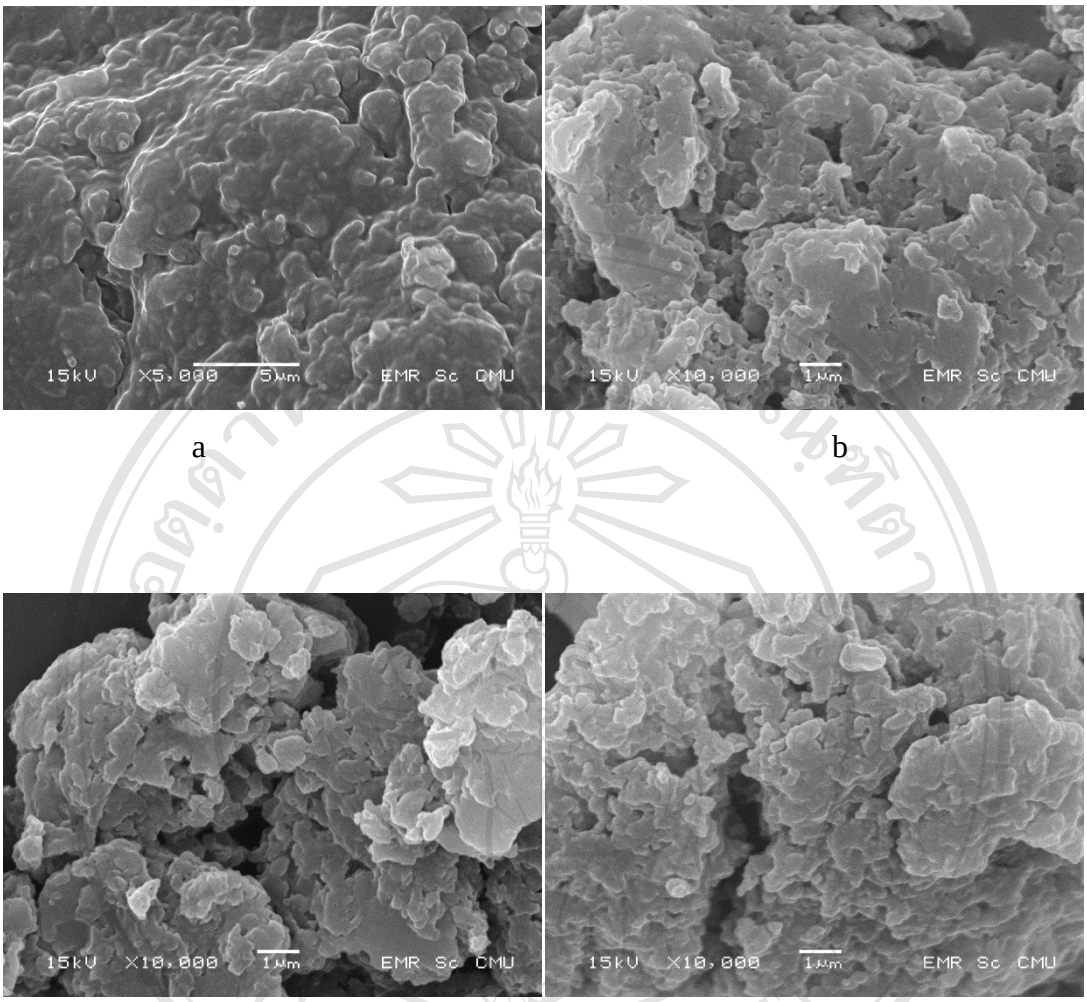
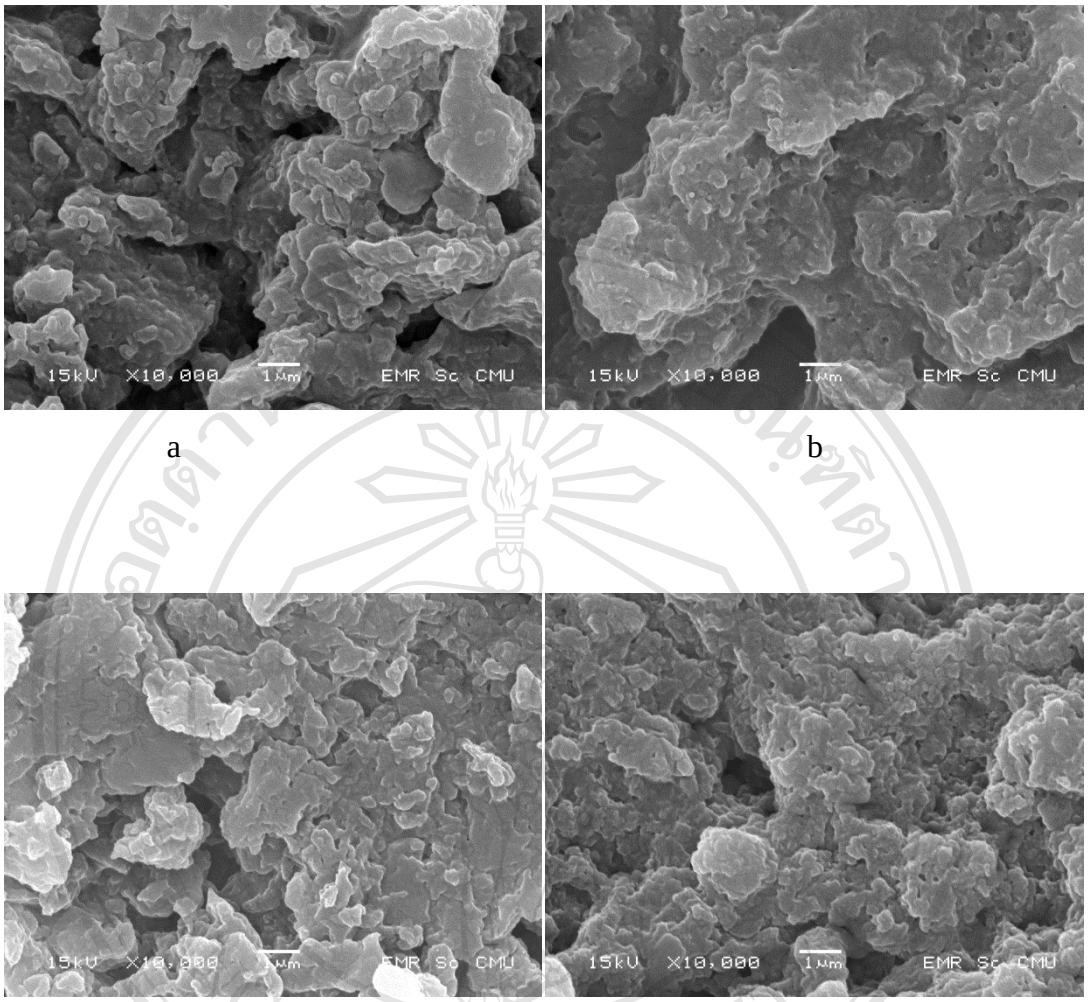


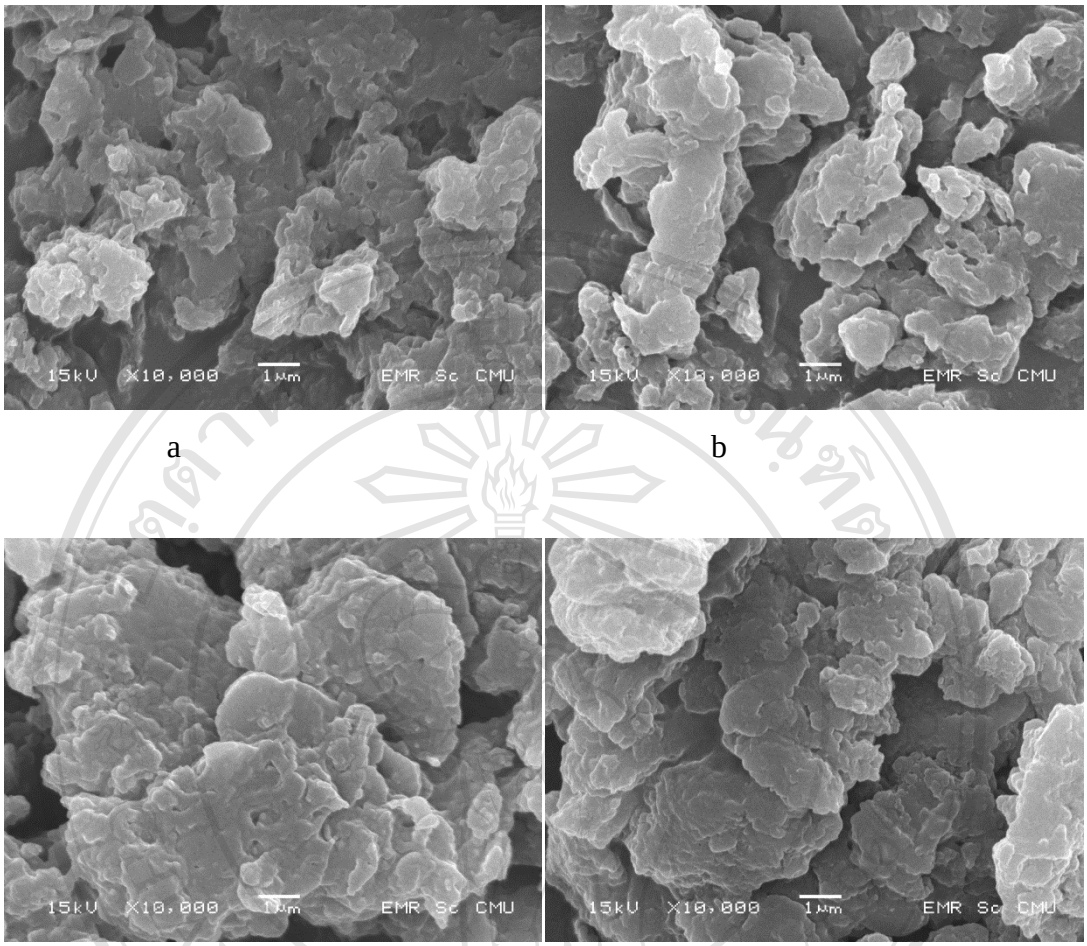
Figure A4 SEM image of 3.0% (w/v) carotenoids encapsulated chitosan (a) without TPP (b) with 0.5% (w/v) TPP (c) with 1.0% (w/v) TPP (d) with 2.0% (w/v) TPP



a b

c d

Figure A5SEM image of 4.0% (w/v) carotenoids encapsulated chitosan
(a) without TPP (b) with 0.5% (w/v) TPP
(c) with 1.0% (w/v) TPP (d) with 2.0% (w/v) TPP



a b

c d

Figure A6 SEM image of 5.0% (w/v) carotenoids encapsulated chitosan (a) without TPP (b) with 0.5% (w/v) TPP (c) with 1.0% (w/v) TPP (d) with 2.0% (w/v) TPP

APPENDIX D

Preparation of sample for analysis of chemical characteristics by Fourier Transform-Infra Red Spectroscopy (FTIR) microscope

Samples analyzed by FTIR microscope were prepared as following;

1. Samples were milled to powder with potassium bromide (KBr).

2. The powder was pressed in a mechanical press to form a translucent pellet through which the beam of the spectrometer could pass.

3. The prepared samples were analyzed by FTIR microscope. The conditions used were as following;

- Number of ample scans: 64
- Number of background scans: 64
- Resolution: 4.000
- Sample gain: 8.0
- Optical velocity: 0.6329
- Aperture: 100.00

APPENDIX E

Calculation of total carotenoids applied as food colorants in salad cream and commercial drink

1. Salad cream

1.1 Calculation of total carotenoids contained in the chitosan-TPP

The absorbance of carotenoids encapsulated chitosan-TPP was 0.7386, the quantity of total carotenoids (x) would calculate as following:

The linear equation was $y = 0.1222x + 0.0044$

$0.7386 = 0.1222x + 0.0044$

$0.7386 - 0.0044 = 0.1222x$

$0.7386 = 0.1222x$

$x = 0.7386/0.1222$

$x = 6.0442$ppm

As 1 ml of ethanol contained total carotenoids of 6.0442 µg, so100 ml Ethanol contained total carotenoids of 6.0442x100 = 604.02 µg. Accordingly, carotenoids encapsulated chitosan-TPP of 0.100 g would contained total carotenoids of 604.02 µg or 0.60402 mg.

FDA (2010) has controlled the maximum level of carotenoids in salad cream and mayonnaise of 155.8 mg total carotenoids per 1 kg food or 155.8 ppm.

In this experiment, the total carotenoids of 133.34 ppm or 4 mg of total carotenoids was applied in 30 g salad cream. As 0.100 g carotenoids encapsulated chitosan-TPP contained 0.6040 mg of total carotenoids or 1 mg of total carotenoids would come from 0.1656 g of encapsulated carotenoids. Thus, 4 mg of total carotenoids would come from 0.6624 g of commercial carotenoids.

1.2 Calculation of total carotenoids contained in the commercial carotenoids (control)

The absorbance of the commercial carotenoids was 0.8533, so the quantity of total carotenoids (x) would calculate as following:

The linear equation was $y = 0.1222x + 0.0044$

$0.8533 = 0.1222x + 0.0044$

$0.8533 - 0.0044 = 0.1222x$

$0.8489 = 0.1222x$

$x = 0.8489/0.1222$

$x = 6.9468$ ppm

As 1 ml ethanol contained total carotenoids of 6.9468 µg, so 100 ml ethanol contained total carotenoids of 6.9468 x100 = 694.68 µg. Accordingly, 0.100 g of commercial carotenoids contained the total carotenoids of 694.68 µg or 0.69468 mg.

In this experiment, 4 mg of total carotenoids was applied in 30 g salad cream. As 0.100 g commercial carotenoids contained 0.69468 mg of total carotenoids or 1 mg of total carotenoids would come from 0.1439 g of commercial carotenoids. Thus, 4mg of total carotenoids would come from 0.5756 g of commercial carotenoids.

2. Commercial drink

2.1 Calculation of total carotenoids contained in the chitosan-TPP

The absorbance of carotenoids encapsulated chitosan-TPP was 0.7363, the quantity of total carotenoids (x) would calculate as following:

The linear equation was $y = 0.1222x + 0.0044$

$0.7363 = 0.1222x + 0.0044$

$0.7363 - 0.0044 = 0.1222x$

$0.7319 = 0.1222x$

$x = 0.7319/0.1222$

$x = 5.9894$ ppm

As 1 ml of ethanol contained total carotenoids of 5.9894 µg, so 100 ml Ethanol contained total carotenoids of 5.9894x100 = 598.94 µg. Accordingly, carotenoids encapsulated chitosan-TPP of 0.100 g would contained total carotenoids of 598.94 µg or 0.59894 mg.

FDA (2010) has controlled the maximum level of carotenoids in drink of15.58 mg of total carotenoids per 1 L or 15.58 ppm.

In this experiment, total carotenoids of 10.00 ppm or 1 mg of total carotenoids was applied in 100 ml commercial drink. As 0.100 g carotenoids encapsulated chitosan-TPP contained 0.59894 mg of total carotenoids or 1 mg of total carotenoids would come from 0.1670 g of encapsulated carotenoids.

2.2 Calculation of total carotenoids contained in the commercial carotenoids (control)

The absorbance of the commercial carotenoids was 0.8287, so the quantity of total carotenoids (x) would calculate as following:

$$\text{The linear equation was } y = 0.1222x + 0.0044$$

$$0.8287 = 0.1222x + 0.0044$$

$$0.8287 - 0.0044 = 0.1222x$$

$$0.8243 = 0.1222x$$

$$x = 0.8243/0.1222$$

$$x = 6.7455 \text{ ppm}$$

As 1 ml ethanol contained total carotenoids of 6.7455 µg, so 100 ml ethanol contained total carotenoids of 6.0110 x100 = 674.55 µg. Accordingly, 0.100 g of commercial carotenoids contained the total carotenoids of 674.55 µg or 0.67455 mg.

In this experiment, 1 mg of total carotenoids was applied in 100 ml commercial drink.As 0.100 g commercial carotenoids contained 0.67455 mg of total carotenoids or 1 mg of total carotenoids would come from 0.1482 g of commercial carotenoids.

APPENDIX F

Physical characteristics of carotenoids encapsulated chitosan

1. Mean diameters and interstitial pore fractions of carotenoids encapsulated in chitosan-TPP matrixes

Table A1 Mean diameters and interstitial pore fractionsof carotenoids encapsulated in chitosan-TPP matrixesat various concentrations of carotenoids and TPP.

Carotenoids (%, w/v)	TPP (%, w/v)	Mean diameters (nm)	Interstitial pore fractions (%)
2.0	0.0	375.235 ± 84.713^{ghi}	14.79 ± 0.57^{j}
	0.5	350.412 ± 96.084^{fgh}	13.17 ± 0.42^{i}
	1.0	329.216 ± 75.669^{cdef}	9.63 ± 0.32^{f}
	2.0	295.118 ± 82.196^{bcde}	8.29 ± 0.61^{e}
3.0	0.0	492.286 ± 84.473^{j}	19.50 ± 0.47^{l}
	0.5	418.917 ± 86.154^{i}	17.27 ± 0.46^{k}
	1.0	341.347 ± 94.900^{efg}	12.28 ± 0.54^{h}
	2.0	317.574 ± 89.764^{cdef}	10.17 ± 0.58^{f}
4.0	0.0	393.152 ± 84.536^{hi}	14.17 ± 0.60^{j}
	0.5	283.714 ± 68.010^{bc}	6.03 ± 0.21^{c}
	1.0	281.071 ± 77.925^{b}	4.96 ± 0.27^{b}
	2.0	230.486 ± 59.267^{a}	3.52 ± 0.83^{a}

Table A1 Mean diameters and interstitial pore fractionsof carotenoids encapsulated in chitosan-TPP matrixesat various concentrations of carotenoids and TPP (continuous)

Carotenoids (%, w/v)	TPP (%, w/v)	Mean diameters (nm)	Interstitial pore fractions (%)
5.0	0.0	499.600±71.478^{j}	17.20±0.64^{k}
	0.5	339.855±86.579^{defg}	11.25±0.06^{g}
	1.0	304.580±76.369^{bcdef}	8.08±0.57^{e}
	2.0	292.912±77.552^{bcd}	7.06±0.22^{d}

Values were mean ± S.D. (n=5). Different letters (a-l) in the same column indicated significant differences ($p \leq 0.05$) between samples.

2. Encapsulation efficiency (%EE)

Table A2 Encapsulation efficiencies of carotenoids in chitosan-TPP matrixes at various concentrations of carotenoids and TPP

Carotenoids (%, w/v)	TPP (%, w/v)	Encapsulation efficiencies (%)
2.0	0.0	39.13±0.39^{a}
	0.5	53.39±0.47^{d}
	1.0	64.92±0.31^{f}
	2.0	75.64±0.37^{j}
3.0	0.0	61.41±0.47^{e}
	0.5	70.18±1.24^{h}
	1.0	72.76±0.69^{i}
	2.0	89.09±0.38^{l}

Table A2 Encapsulation efficiencies of carotenoids in chitosan-TPP matrixes at various concentrations of carotenoids and TPP (continuous)

Carotenoids (%, w/v)	TPP (%, w/v)	Encapsulation efficiencies (%)
4.0	0.0	41.40 ± 1.16^{b}
	0.5	68.84 ± 0.35^{g}
	1.0	70.52 ± 0.22^{h}
	2.0	76.06 ± 0.44^{j}
5.0	0.0	51.45 ± 0.55^{c}
	0.5	69.65 ± 0.50^{gh}
	1.0	73.25 ± 0.21^{i}
	2.0	77.52 ± 0.62^{k}

Values were mean ± S.D. (n=5). Different letters (a-l) in the same column indicated significant differences ($p\leq0.05$) between samples.

3. Color values of carotenoids encapsulated in chitosan-TPP

Table A3 Color values of carotenoids encapsulated in chitosan-TPP at various concentrations of carotenoids and TPP

Carotenoids (%, w/v)	TPP (%, w/v)	L*	a*	b*
2.0	0.0	47.72 ± 0.07^{c}	17.71 ± 0.24^{a}	12.45 ± 0.22^{f}
	0.5	59.12 ± 0.24^{j}	18.10 ± 0.26^{ab}	13.25 ± 0.32^{g}
	1.0	59.23 ± 0.67^{j}	18.43 ± 0.37^{abc}	13.85 ± 0.12^{hi}
	2.0	59.35 ± 0.21^{j}	18.79 ± 0.15^{bcd}	13.90 ± 0.13^{i}

Table A3 Color values of carotenoids encapsulated in chitosan-TPP at various concentrations of carotenoids and TPP

Carotenoids (%, w/v)	TPP (%, w/v)	L*	a*	b*
3.0	0.0	35.14 ± 0.13^{a}	18.45 ± 0.57^{bcd}	11.03 ± 0.13^{e}
	0.5	41.38 ± 0.15^{b}	18.94 ± 0.58^{d}	12.35 ± 0.21^{f}
	1.0	43.04 ± 0.30^{c}	19.44 ± 0.35^{cd}	14.73 ± 0.06^{j}
	2.0	42.91 ± 0.29^{c}	21.76 ± 0.36^{f}	15.50 ± 0.12^{k}
4.0	0.0	48.48 ± 0.55^{e}	19.02 ± 0.06^{cd}	10.32 ± 0.40^{d}
	0.5	59.12 ± 0.15^{j}	19.59 ± 0.57^{cd}	11.31 ± 0.52^{e}
	1.0	57.81 ± 0.51^{h}	20.74 ± 1.17^{e}	13.40 ± 0.85^{gh}
	2.0	59.44 ± 0.63^{j}	21.25 ± 0.75^{e}	14.37 ± 0.40^{j}
5.0	0.0	47.11 ± 0.28^{d}	19.10 ± 0.16^{cd}	7.51 ± 0.18^{a}
	0.5	54.28 ± 0.18^{f}	20.10 ± 0.08^{e}	8.40 ± 0.38^{b}
	1.0	55.81 ± 0.01^{g}	20.80 ± 0.58^{e}	9.61 ± 0.57^{c}
	2.0	58.58 ± 0.68^{hi}	20.94 ± 0.15^{e}	10.99 ± 0.22^{e}

Values were mean ± S.D. (n=5). Different letters (a-j) in the same column indicated significant differences ($p \leq 0.05$) between samples.

4. Solubility of carotenoids encapsulated in chitosan-TPP

Table A4 Solubilities of carotenoids encapsulated in chitosan-TPP at various concentrations of carotenoids and TPP

Carotenoids (%, w/v)	TPP (%, w/v)	Solubility (%)	
		Ethanol	water
2.5% commercial carotenoids; without encapsulation (control)	0.0	78.96 ± 0.20^{e}	77.73 ± 0.39^{f}

Table A4 Solubilities of carotenoids encapsulated in chitosan-TPP at various concentrations of carotenoids and TPP (continuous)

Carotenoids (%, w/v)	TPP (%, w/v)	Solubility (%)	
		Ethanol	water
2.0	0.0	71.21 ± 2.35^{abc}	55.08 ± 1.25^{abc}
	0.5	72.68 ± 2.67^{abcde}	57.32 ± 4.01^{abcd}
	1.0	75.28 ± 3.32^{bcde}	57.75 ± 1.07^{abcd}
	2.0	76.44 ± 2.24^{bcde}	59.13 ± 1.96^{bcde}
3.0	0.0	72.12 ± 1.94^{a}	58.64 ± 1.36^{a}
	0.5	76.35 ± 1.91^{ab}	60.03 ± 1.96^{ab}
	1.0	77.62 ± 1.57^{abcde}	62.24 ± 1.99^{abcde}
	2.0	78.56 ± 5.47^{cde}	63.42 ± 4.70^{cde}
4.0	0.0	68.51 ± 6.47^{abcd}	53.65 ± 2.20^{abcde}
	0.5	70.25 ± 4.40^{bcde}	55.63 ± 3.11^{cde}
	1.0	73.41 ± 2.35^{de}	58.43 ± 0.88^{de}
	2.0	77.42 ± 6.02^{de}	60.20 ± 2.81^{e}
5.0	0.0	68.85 ± 0.83^{a}	54.27 ± 2.58^{ab}
	0.5	73.34 ± 0.85^{abcde}	56.33 ± 4.96^{abc}
	1.0	74.45 ± 1.42^{abcde}	57.65 ± 3.87^{abcd}
	2.0	75.40 ± 2.54^{bcde}	57.92 ± 2.43^{abcd}

Values were mean ± S.D. (n=5). Different letters (a-j) in the same column indicated significant differences ($p \leq 0.05$) between samples.

APPENDIX G

Storage stability of the carotenoids encapsulated in chitosan-TPP

1. Kinetics of antioxidant activities of the carotenoids encapsulated in chitosan-TPP during storage at 5, 25 or 40°C for 60 days.

Table A5 The values of R-square obtained from the trend lines of zero order, first order and second order plots of percentage $ABTS^{+}$ inhibition of encapsulated carotenoids during storage at 5, 25 or 40°C for 60 days.

Temperature (°C)	R-square (R^2)		
	Zero order	First order	Second order
5	0.9919	0.9923	0.9913
25	0.9899	0.9911	0.9832
40	0.9776	0.9951	0.9730

2. Kinetics of color values of carotenoids encapsulated in chitosan-TPP during storage at 5, 25 or 40°C for 60 days.

Table A6 The values of R-square obtained from the trend lines of zero order, first order and second order plots of L* value of encapsulated carotenoids during storage at 5, 25 or 40°C for 60 days.

Temperature (°C)	R-square (R^2)		
	Zero order	First order	Second order
5	0.9935	0.9943	0.9939
25	0.9949	0.9956	0.9947
40	0.9943	0.9953	0.9940

Table A7 The values of R-square obtained from the trend lines of zero order, first order and second order plots of a* value of encapsulated carotenoids during storage at 5, 25 or 40°C for 60 days.

Temperature (°C)	R-square (R^2)		
	Zero order	First order	Second order
5	0.9808	0.9820	0.9811
25	0.9747	0.9788	0.9769
40	0.9925	0.9937	0.9870

Table A8 The values of R-square obtained from the trend lines of zero order, first order and second order plots of b* value of encapsulated carotenoids during storage at 5, 25 or 40°C for 60 days.

Temperature (°C)	R-square (R^2)		
	Zero order	First order	Second order
5	0.9859	0.9873	0.9848
25	0.9967	0.9980	0.9915
40	0.9888	0.9924	0.9842

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