APPENDIX A
METHOD DEVELOPMENT FOR DETERMINATION OF ETHANOL IN DISTILLED LIQUORS BY NEAR INFRARED SPECTROMETRIC FLOW INJECTION ANALYSIS

1. Introduction

1.1 General

Thai legislation : [48] defines alcohol beverages as those contain alcoholic strength or ethyl alcohol content not more than 60 degrees (% v/v) and classifies into two types; fermented liquors and distilled liquors or distilled spirits. The fermented liquors process from fermentation of fruits or grains without distillation, whareas the distilled liquors process from the distillation of fermented product, and then aging, blending and flavoring as needed. The official methods [49] for determination of ethanol by volume in distilled liquors are by using pycnometer, hydrometer, densitometric method, refractometer or William's Field test. Those methods are long time consuming for analysis. Alternative methods that can be modified for determination of ethanol content are such as Headspace-GC-FID and SPME-GC-FID [50], but they are high cost. Thus, the flow injection combined with near infrared spectrometer method for determination of ethanol in distilled liquors was developed. It is based on the combination bands associated with O-H streteh and C-H streteh in the near-infrared (NIR) region. This method proposed for the routine analysis which could minimize the sample preparation, analysis time and reagent consuming.

1.2 Near Infrared Spectroscopy [51,52]

Near-IR region was first discovered by William Herschel in 1800 and often referred to as the Herschel region [53]. The near infrared (NIR) region of the spectrum extends from 770 nm to 3000 nm (13,000 $cm^{-1}$to 3300 cm^{-1}). In general, the origin of near-IR is the overtones and combination bands of fundamental vibrations in

the mid-infrared spectrum, thus the molar absorptivities are low and detection limits are on the order of 0.1%. Because of the low sensitivity of near infrared, high S/N operation is a necessity. According to the energetic self-limiting factor, not all of the overtones or combinations are observed. Only the result of vibrations of light atoms that have strong molecular bonds such as hydrogen attached to nitrogen, oxygen or carbon (NH, OH, and CH) are predominant. Even though the vibrational band assignment for molecules is not known as well as the traditional IR band assignments, it is possible to simplify the process of band assignment by making some accurate assumptions such a C-H assumption as:

$$N+1 \text{ Overtone spectrum} = \text{C-H stretch} + N^{th} \text{ Overtone spectrum} * \text{Anh}$$

where Anh. is the frequency anharmonicity of the C-H stretch [54].

Moreover, it is common to ignore the much weaker combination and overtone bands. To first order, all NIR spectra consist only of those bands which include at least one quantum of hydrogen stretch. Thus, the chemical structures are limited to simply observe.

In spite of the selectivity of this region, it permits considering absorptions from the analyte in a specific part of the spectrum that are not affected by the absorptions of a molecule of the matrix or possible interferences. Near infrared rates in selectivity less than that of mid-infrared, however, it exceeds that of ultraviolet, visible and far-infrared spectroscopy. Since the weak bands in the near-infrared are broad and overlapping, resolution is usually not a problem but reproduction of the same wavelength is essential for quantitative analysis and such bands can often be determined with accuracy and precision.

Instrumentation for the near-infrared region is similar to that used for UV/visible spectroscopy. The most common of the near-IR analyzers in use are grating monochromator instruments equipped with a quartz tungsten halogen source and a PbS detector [55]. Cells usually used are quartz or glass with a path length vary from 0.1 to 10 cm. Near-IR spectroscopy is a viable technique which can be applied to many fields such as agriculture, foods and pharmaceutics, since it offers four principal advantages: speed, simplicity of sample preparation, multiplicity of analyses from a single spectrum, and the intrinsic nonconsumption of the sample. However, it has some disadvantages that it is insensitive to minor constituents (less than 1%)

unless combining with wet chemistry. According to the wide applicability, the possibility of in situ applications without sample pretreatment and the availability of multivariate statistical methods for data analysis, the near-IR can be used as a detector of FIA. In fact, only a few literatures reported the utilization of near-IR in FIA: for continuous-flow based on the single wavelength [56] and sequential wavelengths using acoustooptic tunable filter (AOTF) based instrument [57]. The purpose of this study is to apply the near-IR as detector in FIA for the determination of ethanol in distilled liquors.

2. Experimental

2.1 Instruments and apparatus

1. Peristaltic pump: Eyela MP-3; Tokyo Rikakikai, Japan.
2. Six port injection valve; Upchurch, USA.
3. Home-made flow through cell
4. UV/VIS/NIR spectrometer: Lamda 19; Perkin Elmer, USA. (Double-beam, double monochromator, grating with 360 lines mm^{-1}, equipped with a tungsten-halogen lamp source and PbS detector) consisting of
   a. Processor: Compaq presario 460; Compaq, USA. (with a UVCSS operating software)
   b. Printer: Deskjet 520; Hewlett Packard, USA.
5. Vacuum evaporator: Rotavapor R-124; Buchi, Switzerland.
6. Analytical balance: PM400; Mettler, Switzerland.
7. Gas chromatography: Star3400CX; Varian, Australia., consisting of
   a. (SPME) autosample, 65μ carbowax/divinylbenzene: Varian, Australia.
   b. Capillaly column, 100% dimethylpolysiloxane (volatile, 30m ×0.53mm): RTX; Varian, Australia.
   c. Flame ionization detector

2.2 Chemicals

1. Sodium chloride: NaCl, A.R grade; BDH, England.
2. Calcium chloride anhydrous: $CaCl_2$; BDH, England.
3. Powdered type 5A molecular sieve

4. Ethanol 96%: C_2H_5OH, commercial grade; Thailand.

5. Chloroform: $CHCl_3$, commercial grade.

6. Isopropanol: C_3H_7OH, A.R grade; E.merck, Germany.

7. Ethanol absolute: C_2H_5OH, A.R grade; E.merck, Germany.

2.3 Solutions

1. Dried chloroform

500 ml of Chloroform was washed with water to remove impurity ethanol, dried for several hours over $CaCl_2$ anh. and then distilled using vacuum evaporator (40°C,473 mbar). Powdered type 5A molecular sieve (oven at 220°C ,8 hrs) was put in the distillate during using.

2. Internal standard solution

Internal standard solution was prepared by diluting isopropanol with deionized water to obtain accurate concentration of about 0.25%V/V

3. Ethanol standard solutions

Ethanol absolute was diluted with deionized water to obtain accurate concentration range 0-400mg%

4. Sample preparation (for GC)

Sample was transferred to a 100 ml volumetric flask and diluted with deionized water to give concentration in the range of calibration curve of 0-400mg%

2.4 Procedure

2.4.1 Determination of ethanol in distilled liquors by near infrared spectrometric flow injection analysis.

Distilled liquor sample or ethanol standard solution was extracted with dried chloroform for 1 min. Then the extract was injected into the stream of dried chloroform and then introduced to the home-made flow cell in the FI-NIR manifold. The absorbance of the stream was continuously monitored either at 2305 or 2636 nm. The ethanol content in sample evaluated from interpolating the peak height values obtained on the calibration curve.

2.4.2 Determination of ethanol in distilled liquors by automated SPME-GC-FID

The 100-µl standard solution and sample preparation were transferred and mixed with 100-µl internal standard solution in 2-ml vial, then sorbed by SPME

before injecting into dimethylpolysiloxane capillary column using N_2 as carrier gas with the flow rate of 12 min/ml and split ratio of 20:1 and detected with FID. The quantity of ethanol in the sample can be taken by interpolating the peak areas obtained on the calibration curve.

3. Results and discussion

3.1 NIR absorption spectra

The NIR absorption spectra of the extracted ethanol were investigated. Extraction as carried out in separating funnel by mixing 20-ml ethanol standard solution in different concentrations [range 30%-50%V/V] with 10-ml of dried chloroform for 1 min. The chloroform layer was then separated and directly introduced into the 1-mm path length home made flow cell by fia manifold as shown in Figure A.1, which injection valve was switched to loading position.

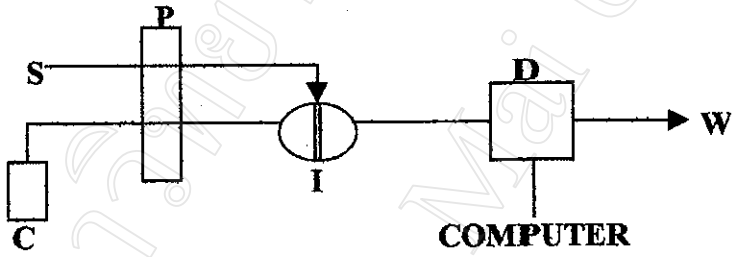


Figure A.1 Fia manifold for ethanol determination; C: carrier stream, S: sample, P: peristaltic pump, I: six-port injection valve, D: UV/VIS/NIR spectrometer, W: waste

The spectra of the extracted ethanol and dried chloroform were recorded from 2000-2800 nm using air with the empty flow cell as reference, as shown in Figure A.2. The differences of ethanol spectral absorption bands from chloroform are centered at 2074, 2268, 2305 , 2464 and 2636 nm due to the combination bands associated with O-H and C-H vibrational transitions in mid infrared regions. The band assignment of ethanol in NIR region correlated to its infrared spectra are illustrated in Table A.1 and A.2. It was found that the spectral lines of mid infrared correlated to their combination bands as well. Since there are a large number of possible combination bands, a moderate or higher correlation might be selected [58]. Moreover, it was found that sodium chloride added to decrease emulsion occur during

the extraction has no effect to the ethanol spectra but increases their intensities as shown in Figure A.3. The linear regressions, correlation coefficients and molar absorptivities obtained in various absorption bands are illustrated in Table A.3 and Figure A.4. The results show that the correlation with respect to the series of ethanol at the selected wavelengths exceed by 0.9 which are good candidates for quantitative analysis. In order to study the absorption bands representing of those due to the combination bands, the bands at 2305 and 2636 nm, respectively, were selected.

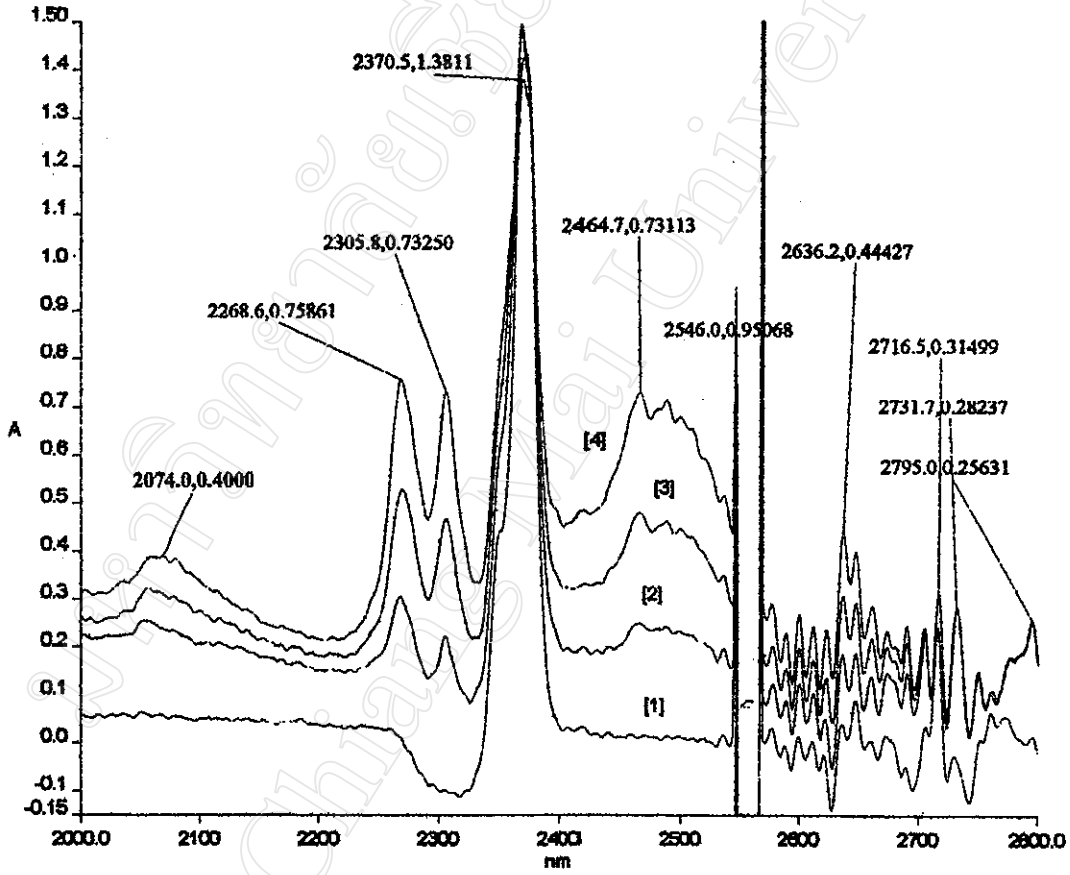


Figure A.2 NIR spectra of ethanol in dried chloroform. Ethanol concentrations: [1] dried chloroform; [2] 30%V/V; [3] 40%V/V and [4] 50%V/V

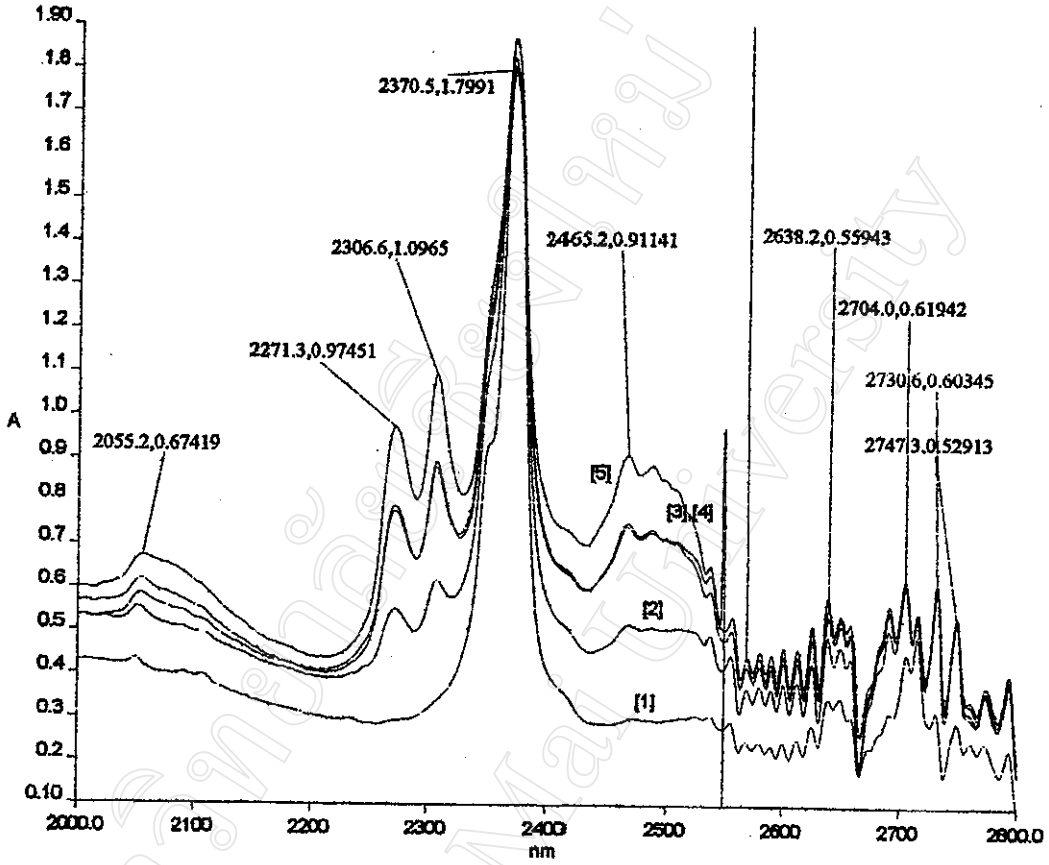


Figure A.3 NIR spectra in dried chloroform (NaCl added). [1] dried chloroform; [2] diluted clear distilled liquor (20%V/V labelled); [3] 40% ethanol standard solution; [4] colored distilled liquor(40%V/V labelled, no NaCl added) and [5] colored distilled liquor (40%V/V labelled)

Table A.1 Infrared spectrum interpretation of ethanol [59,60,61]

Wavenumber (cm^{-1})	Assignment
3335	O-H stretch [ν(OH)]
2985,2898	C-H aliphatic stretch [ν(CH)]
1428	CH_2 scissoring [$\delta(CH_2)$]
1388	CH_3 symmetric bending [$\delta_s(CH_3)$]
1082,1050	C-O stretch [ν(CO)]
880	CH_2 rocking [$\rho_r(CH_2)$]

Table A.2 Near infrared band assignments of ethanol

assignment	Wavenumber (cm^{-1})	Calculated wavelength (nm)	Actual wavelength (nm)	Relative error (nm)
$\nu(OH)+\delta(CH_2)$	3335+1428	2099	2074	25
$\nu(OH)+\nu(CO)$	3335+1082	2264	2268	4
$\nu(OH)+\nu(CO)$	3335+1050	2280	2305	25
$\nu(CH)+\nu(CO)$	2985+1050	2478	2464	14
$\nu(CH)+\rho_r(CH_2)$	2898+880	2646	2636	10

Table A.3 NIR absorption of the series of ethanol (23.7-39.5%W/V) obtained from various absorption bands.

Ethanol [%W/V]	Absorbance				
	2074 nm	2268 nm	2305 nm	2464 nm	2636 nm
23.7	0.2571	0.3054	0.2000	0.2414	0.1868
31.6	0.3286	0.5293	0.4621	0.4805	0.3102
39.5	0.4000	0.7586	0.7325	0.7311	0.4443
$y = ax + b$	$y = 0.009x$ -0.043	$y = 0.029x$ -0.375	$y = 0.034x$ -0.6	$y = 0.031x$ -0.495	$y = 0.016x$ -0.201
r^2	1.000	0.999	0.999	0.999	0.999
Molar absorptivity $cm^{-1}M^{-1}$	0.42	1.34	1.57	1.43	0.74

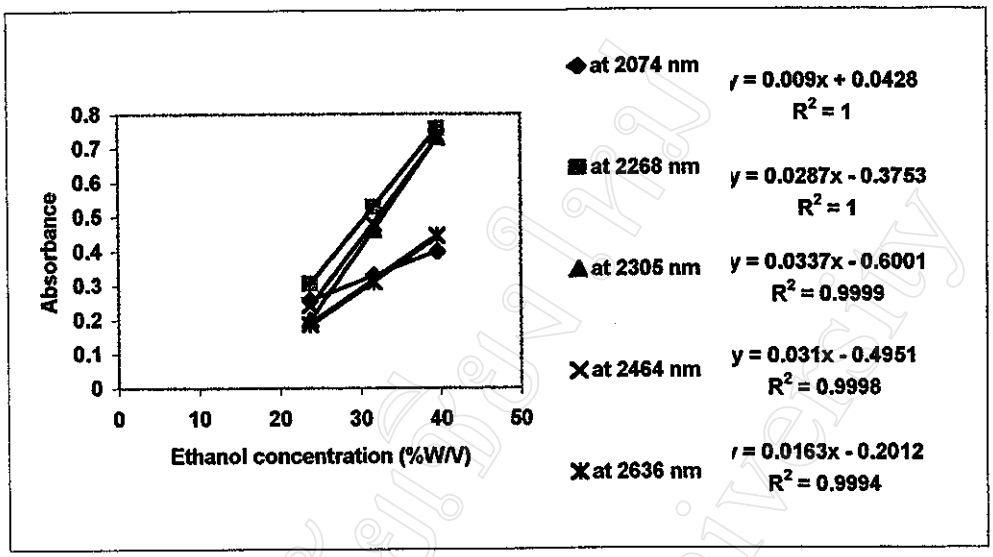


Figure A.4 Study of ethanol NIR absorption bands.

3.2 Optimization of FI parameters and NIR parameters

Preliminary conditions were used as following:

Carrier solution	dried chloroform
Flow rate of carrier solution	2 ml/min
Flow through cell pathlength	1 mm
NIR parameters (UVCSS operating software)	
Time interval	0.3 sec
Measurement wavelength	2636 nm

For optimization of fi parameters, the effect of sample volume and carrier flow rate were investigated. According to the NIR spectrometry was carried out at a fix wavelength and automatically adjusting slit width, the time interval was only parameter which could be evaluated. The different concentrations of ethanol (20%-50%V/V) were extracted as described in 3.1 and the chloroform layer was injected into the stream of dried chloroform of fi-NIR system as shown in Figure A.1. The results are shown in Tables A.4-A.6 and Figures A.5-A.7. It was found that the much more sample volume, the higher peak height obtained. The peak height of a large sample volume decreases due to the doublet peaks performed because of high dispersion. In order to decrease the corresponding time and increase the sensitivity, a sample volume of 100 μl was selected. The carrier flow rate of 2.0 ml/min is considered, according to the higher sample throughput (240 injections per hour) and

the less carrier solution used. The optimum time interval is 0.3 sec which gives the highest sensitivity and correlation coefficient due to the well defined fi peaks provided.

Table A.4 Effect of sample volume on peak height; mean of triplicate injections.

Ethanol (%V/V)	Peak height (cm)		
	50 μl	100 μl	200 μl
20	1.1	1.7	2.1
30	2.4	4.5	3.9
40	4.1	6.5	6.4
50	6.0	8.7	8.4
$y = ax + b$	$y = 0.16x - 2.34$	$y = 0.23x - 2.7$	$y = 0.21x - 2.29$
r^2	0.993	0.995	0.996

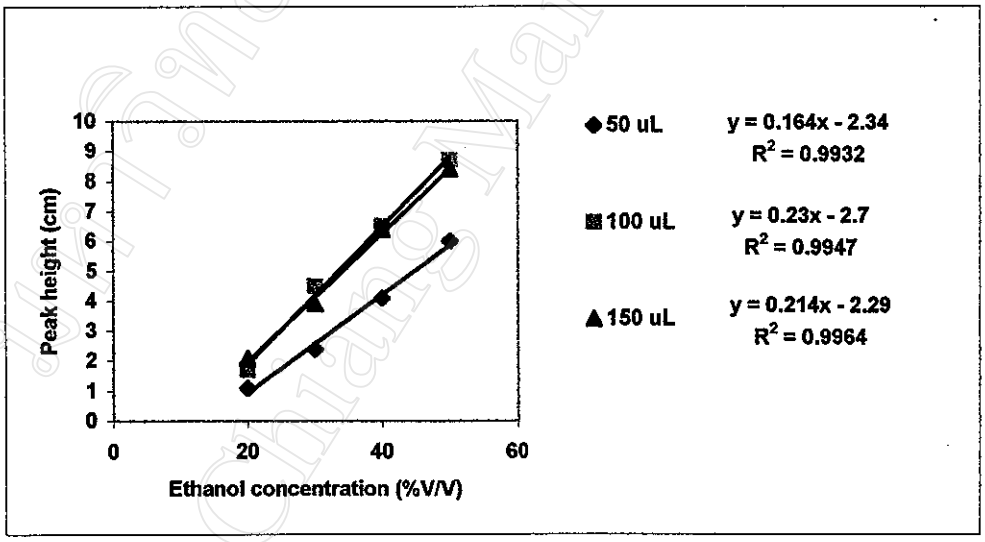


Figure A.5 Effect of sample volume on peak height.

Table A.5 Effect of carrier flow rate on peak height; mean of triplicate injections.

Ethanol (%V/V)	Peak height (cm)		
	2.0 ml/min	3.5 ml/min	5.0 ml/min
20	1.7	1.6	1.5
30	4.5	4.9	5.3
40	6.5	6.7	7.4
50	8.7	8.0	8.7
$y = ax + b$	$y = 0.23x - 2.7$	$y = 0.21x - 2.05$	$y = 0.24x - 2.57$
r^2	0.995	0.954	0.946

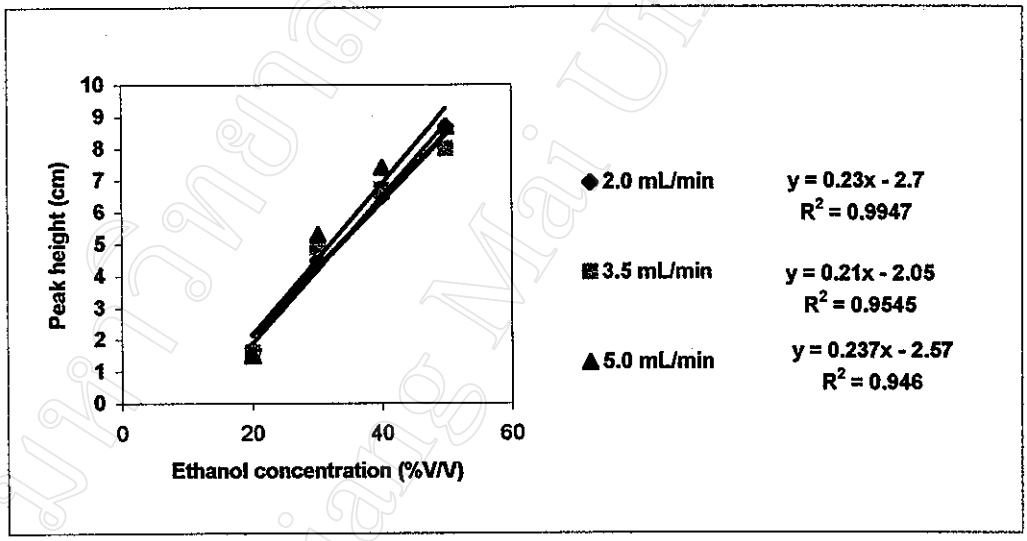


Figure A.6 Effect of carrier flow rate on peak height.

Table A.6 Effect of time interval on peak height; mean of triplicate injections (sample volume of 100 μl and carrier flow rate of 2.0 ml/min)

Ethanol (%V/V)	Peak height (cm)		
	0.3 sec	0.5 sec	1.0 sec
20	1.7	1.4	1.3
30	4.5	3.9	3.3
40	6.5	5.6	4.8
50	8.7	7.8	6.0
$y = ax + b$	$y = 0.23x - 2.7$	$y = 0.21x - 2.64$	$y = 0.16x - 1.61$
r^2	0.995	0.995	0.987

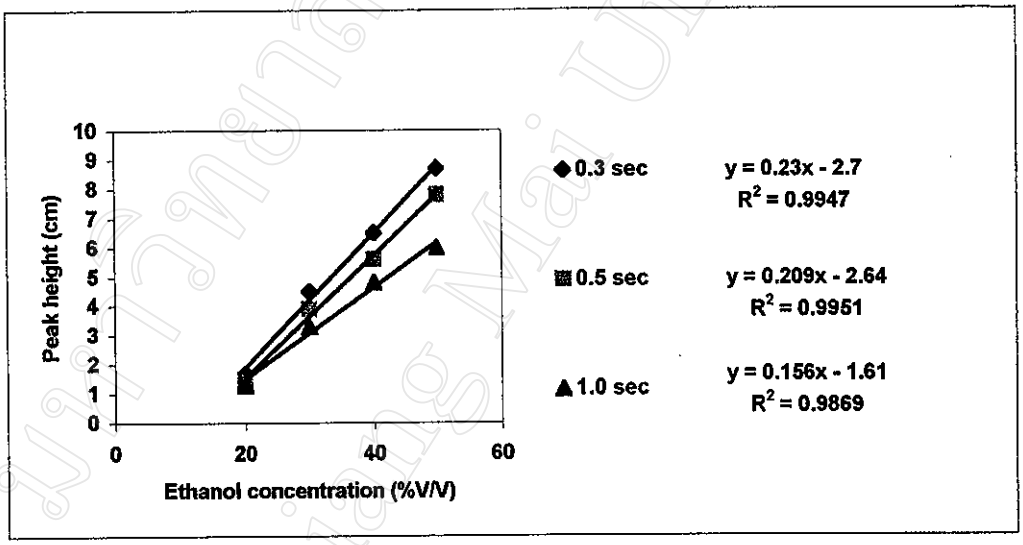


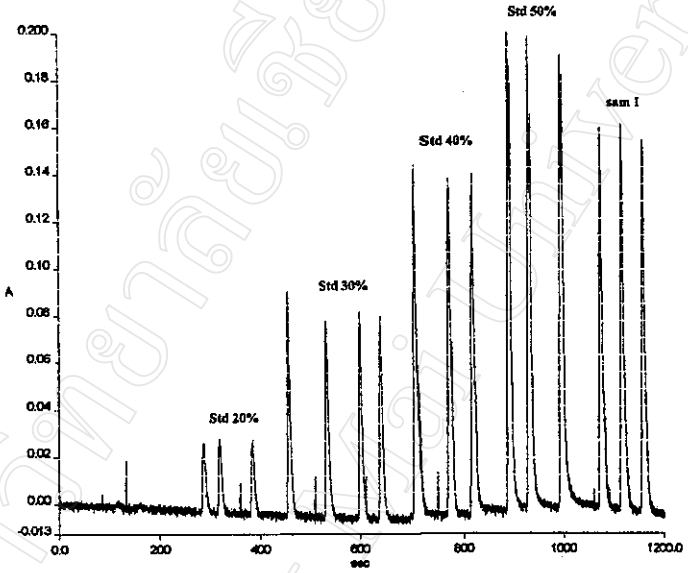
Figure A.7 Effect of time interval on peak height.

The optimum conditions for recommended fi-NIR system are summarized as following:

Carrier solution	dried chloroform
Flow rate of carrier solution	2.0 ml/min
Sample volume	100 μl
Flow through cell path length	1 mm
NIR parameter	
Time interval	0.3 sec

3.3 Calibration curve and detection limit

The optimum fi-NIR system described in 3.2 was used. The calibration curve and detection limit of the conditions used at both wavelengths of 2305 and 2636 nm were investigated. The results are shown in Figure A.8 and Figure A.9. The results are obtained in the linear range of 20%-50%V/V of ethanol in both wavelengths of 2305 and 2636 nm with the detection limit of 1%V/V and 5%V/V, respectively.



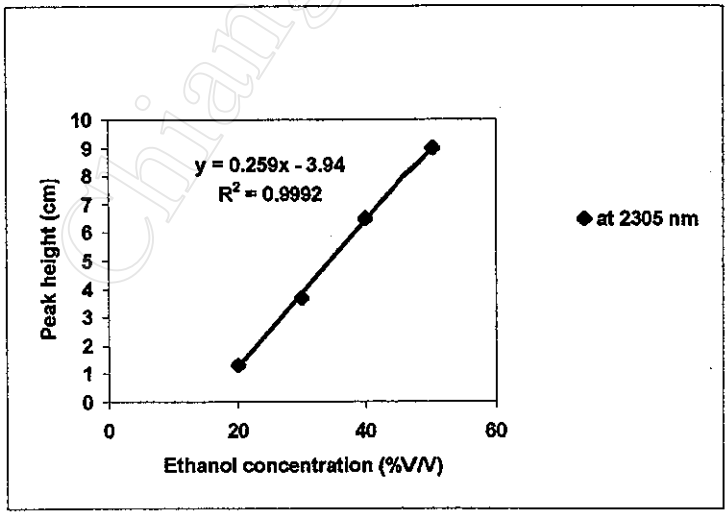


Figure A.8 (a) Fi signal of ethanol calibration at 2305 nm
(b) Calibration curve

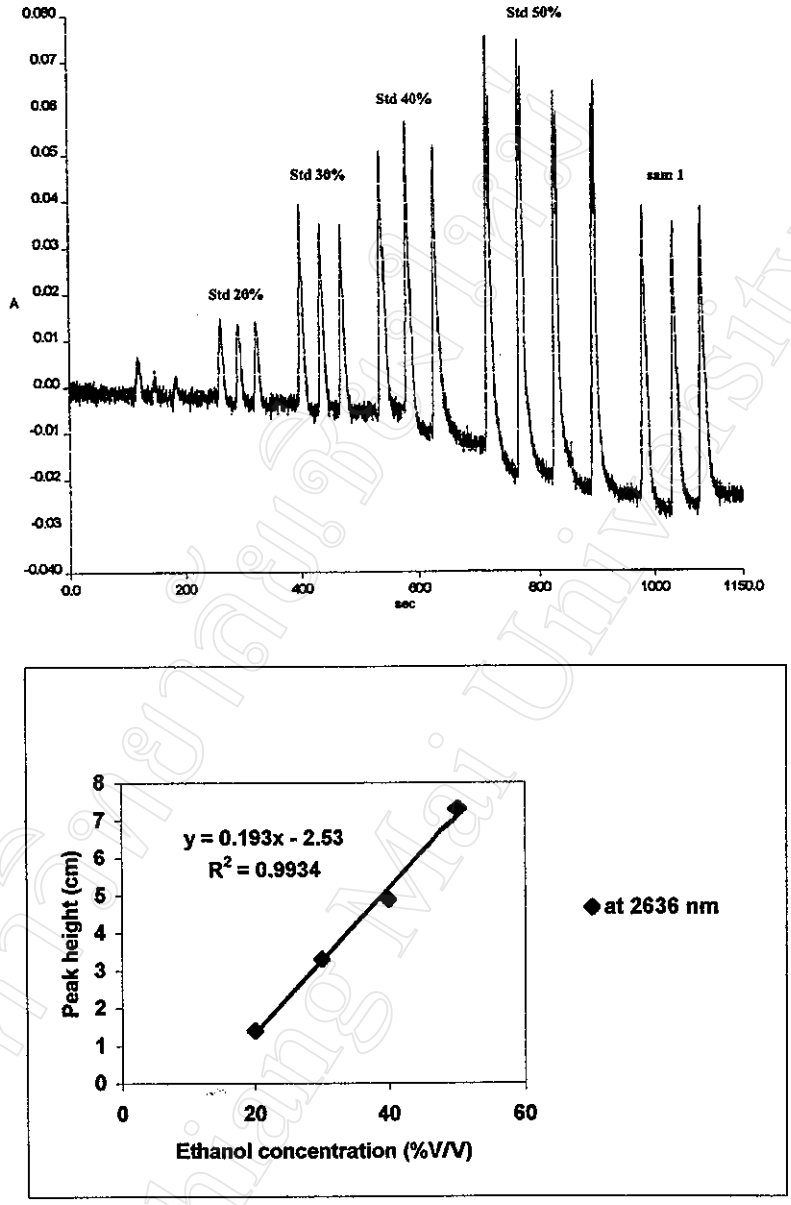


Figure A.9 (a) Fi signal of ethanol calibration at 2636 nm

(b) Calibration curve

3.4 Precision of fi-NIR system and reproducibility of the method

The studies were carried out by using the optimum fi-NIR system described in 3.2 in both measurement wavelengths of 2305 and 2636 nm with two distilled liquor samples; clear and colored one. The calibration curve range of 20-40%V/V was used. The precision was determined by 11 replicate injections of each sample, whereas the reproducibility of the method was evaluated by triplicate injections of five independent measurement of each sample. The results are shown in Table A.7

injections of five independent measurement of each sample. The results are shown in Table A.7

Table A.7 The precision of FI-NIR system and reproducibility of the method

sample	Precision (%RSD,n=11)		Reproducibility (%RSD,n=5)	
	At 2305 nm	At 2636 nm	At 2305 nm	At 2636 nm
Clear sample (30%V/V)	4.2	2.8	4.6	3.2
Colored sample (25%V/V)	2.7	3.3	6.4	7.8

3.5 Study of percentage recoveries

Percentage recoveries of ethanol by fi-NIR was investigated at the wavelength of 2636 nm by dissolving known amounts of ethanol standard solution in two distilled liquor samples; clear (18.9%V/V ethanol) and colored one (18.5%V/V ethanol) with the optimum conditions as described in 3.2. The percentage recoveries between 96-103 for the clear sample and between 84-122 for the colored sample were obtained as shown in Table A.8.

Table A.8 Percentage recoveries of ethanol

sample	Standard added (%V/V)	Standard found (%V/V)	% recovery
Clear sample (18.9%V/V)	5.0	4.8	96.0
	10.0	10.3	103
	15.0	15.4	103
	20.0	19.4	97.0
Colored sample (18.5%V/V)	5.0	4.2	84.0
	10.0	10.7	107
	15.0	18.3	122
	20.0	24.4	122

3.6 Determination of ethanol in distilled liquor samples

The optimized fi-NIR system was applied to the determination of ethanol in distilled liquor samples. The 20-ml of sample was transferred into the 100-ml separating funnel, added with 10-ml dried chloroform and extracted for 1 min. The

chloroform layer was separated and then injected into the system. The results obtained were compared with the ones determined by automated SPME-GC-FID as shown in Table A.9. The t-test values are 0.74 and 1.72 for the measurement wavelength of 2305 nm and 2636 nm, respectively, while the tabulated t for 18 degrees of freedom at the 95% confidence level is 2.10. Thus there are no significant difference in the results by the two methods.

Table A.9 Determination of ethanol in various distilled liquors

sample	Labelled amount (%V/V)	Fi-NIR at 2305 nm		Fi-NIR at 2636 nm		SPME-GC-FID	
		Amount found (%V/V)	%L.a.*	Amount found (%V/V)	%L.a.	Amount found (%V/V)	%L.a.
1.vodka	40.0	38.2	95.5	39.5	98.8	40.9	102.2
2.vodka	40.0	38.2	95.5	40.8	102.0	38.2	95.5
3.vodka	37.5	36.9	98.4	40.2	107.2	37.7	100.5
4.gin	37.5	37.0	98.7	38.5	102.7	38.6	102.9
5.gin	40.0	39.8	99.9	40.6	101.5	42.2	105.5
6.rice whisky	35.0	36.9	105.4	37.3	106.6	35.6	107.7
7.brandy	38.0	45.4	119.5	47.8	125.8	41.3	108.7
8.whisky	40.0	44.6	111.5	42.8	107.0	39.7	99.2
9.whisky	40.0	45.1	112.7	45.0	112.5	42.3	105.8
10.whisky	35.0	40.8	116.6	41.9	119.7	36.5	104.3

* % Labelled amount = $\frac{\text{amount found}}{\text{labelled amount}}$ X 100

4. Conclusion

The determination of ethanol by near infrared spectrometric flow injection analysis (FI-NIR) has been developed. It is based on the combination bands due to the O-H stretch and C-H stretch in near infrared region of 2000-2800 nm. According to these weak bands are more subject to their environment by affecting the frequency shifts and amplitude changes than the fundamental bands, they provide selective and quantitative analysis. The ethanol absorption bands centered at 2074, 2268, 2305, 2464 and 2636 nm can be selected for monitoring since all of them give good

correlation coefficient. For this study, the absorption bands at 2305 and 2636 nm are selected for studying models. The ethanol in sample was extracted into dried chloroform layer which then was injected into the FI-NIR system. The optimum conditions used were shown in 3.2. The concentration range for the measurements at 2305 and 2636 nm, conforming to Beer' law are 20%-50%V/V of ethanol with the detection limit of 1%V/V and 5%V/V, respectively. At 2305 nm, the precision for the clear (30%V/V ethanol) and colored (25%V/V ethanol) distilled liquor samples of 4.2% RSD (n=11) and 2.7% RSD (n=11),respectively, were found. The reproducibility of the methods were found to be 4.6% RSD (n=5) and 6.4% RSD (n=5),respectively. At 2636 nm, the precision for the clear and colored ones of 2.8% RSD (n=11) and 3.3% RSD (n=11), respectively, were found. The reproducibility of the methods were found to be 3.2% RSD (n=5) and 7.8% RSD (n=5), respectively. The percentage recoveries were found to be 96-103 for the clear sample and 84-122 for the colored sample.

The method has been applied to the determination of ethanol in distilled liquors. The results obtained are no significant difference from those obtained by SPME-GC-FID at the 95% confidence level. The developed near infrared spectrometric flow injection system provides a precise, simple, rapid with the sample throughputs of 240 injections per hour and economic method which requires minimal sample pretreatment and is easily applied in routine work.

APPENDIX B

MOLAR ABSORPTIVITY EVALUATION

The calculation of molar absorptivity (ε) is as following: [45]

The fundamental equation of the Beer-lambert law can be stated as:

$$A = \varepsilon b\, c$$

Where, A = absorbance

ε = molar absorptivity or molar absorption coefficient

($cm^{-1}.mol^{-1}.l$ or $cm^{-1}.M^{-1}$)

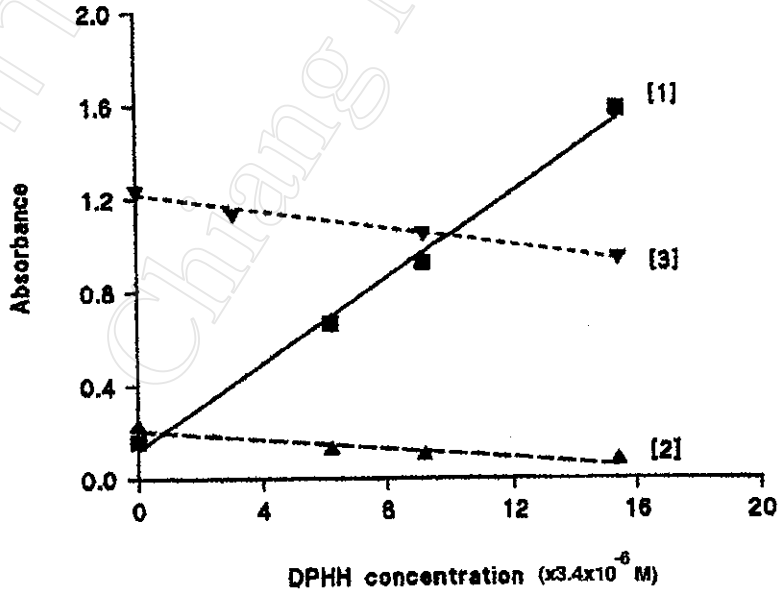
b = cell path length = 1 cm

c = concentration (M)

or $\varepsilon = A/bc$ $(cm^{-1}.M^{-1})$

= slope/b $(cm^{-1}.M^{-1})$

For example in Figure 3.4:



For (1), $\varepsilon = \underline{0.092}$

$3.4X10^{-6}$

$= 2.7X10^{4}\ cm^{-1}.M^{-1}$

APPENDIX C

DETECTION LIMIT EVALUATION

The detection limit was described by Miller and Miller [46] as following:

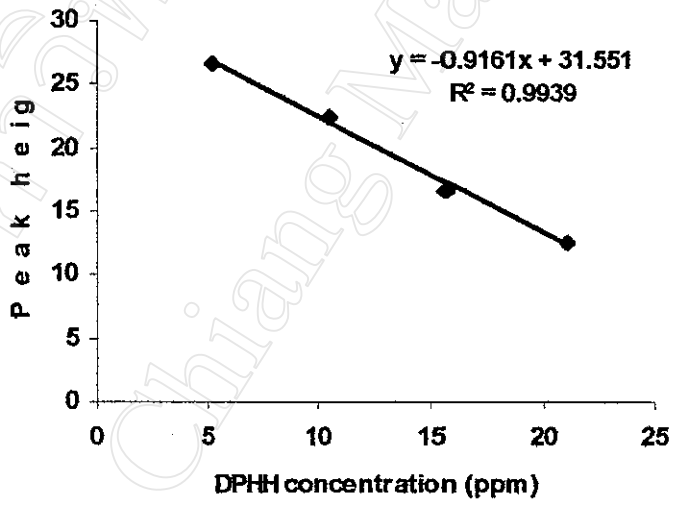
1. Definition

The detection limit is "the analyte concentration giving a signal equal to the blank signal, y_B, plus three standard deviations of the blank, s_B".

$$y = y_B + 3s_B$$

2. Calculation

For example, the detection limit calculation of diphenhydramine hydrochloride in the concentration range 5.2-21.0 ppm.(data from Table 3.7)



$$y = y_B + 3s_B = 31.55 + 3s_B \qquad (1)$$

From a regression line equation;

$$y = b(x) + a = -0.916(x) + 31.55 \qquad (2)$$

When, a, b = intercept and slope of regression line

r = correlation coefficient

The data is shown in a table as following;

x_i	y_i	$\hat{y}_i$	$\lvert y_i - \hat{y}_i \rvert$	$\lvert y_i - \hat{y}_i \rvert^2$
5.2	26.6	26.79	-0.19	0.04
10.5	22.5	21.93	0.57	0.32
15.7	16.6	17.17	-0.57	0.32
21.0	12.5	12.31	0.19	0.04
				$\Sigma = 0.72$

y_i = the observed value correspond to the x_i

$\hat{y}_i$ = the point on the calculated regression line corresponding to the individual x_i values or y_i = -0.916(x)+31.55

The statistic s_B is calculated by:

$$s_B = \left[\left(\sum_i (y_i - \hat{y}_i)^2 \right) / (n-2) \right]^{1/2}$$

$$= (0.72/2)^{1/2}$$

The s_B = 0.6, then is inserted into the equation (1):

$$y = 31.55+3(0.6) = 32.15$$

From the equation (2):

$$32.15 = -0.916(x)+31.55$$

$$x = 1.0$$

Hence, the detection limit of diphenhydramine hydrochloride in the range of 5.2-21.0 ppm using 1.054X10^{-4}M bromocresol green as ion pair formation reagent is 1.0 ppm. As obvious calculation, the detection limit of diphenhydramine hydrochloride in the range of 75.1-187.8 ppm using 5.42X10^{-4}M bromocresol green is 15.3 ppm.

APPENDIX D
COMPOSITION OF SAMPLES

The active ingredients in each sample used in this study are as following:

1. Benadryl®

Each capsule contains:

Diphenhydramine HCl	25.0 mg

2. Benadryl®Cough Syrup

Each 5ml contains:

Diphenhydramine HCl	12.5 mg
Ammonium chloride	125.0 mg
Sodium citrate	50.0 mg
Menthol	1.0 mg
Alcohol	5%

3. Cotussin®

Each 5ml contains:

Diphenhydramine HCl	12.5 mg
Ammonium chloride	125.0 mg

4. Coldanyl®Expectorant cough syrup

Each 5ml contains:

Diphenhydramine HCl	12.5 mg
Ammonium chloride	125.0 mg
Sodium citrate	50.0 mg

5. Bronchoprex®

Each 5ml contains:

Diphenhydramine HCl	10.0 mg
Sodium citrate	40.0 mg
Dextromethorphan HBr	7.5 mg
Menthol	1.0 mg

Citric acid syrup q.s.ad	5 ml

6. Bronchoprex®

Each 5ml contains:

Diphenhydramine HCl	4.0 mg
Bromhexine HCl	4.0 mg
Glyceryl gauiacolate	50.0 mg

7. Caladryl® cream

$1\%^{w}/_{w}$ Diphenhydramine HCl

$8\%^{w}/_{w}$ Calamine

$0.1\%^{w}/_{w}$ Camphor

8. Caldamine® lotion

Each ml contains:

Calamine	100.0 mg
Zinc oxide	30.0 mg
Diphenhydramine HCl	10.0 mg
Camphor	15.0 mg
Menthol	5.0 mg
Ethyl alcohol 95%	0.05 ml

9. Cadramine-v® lotion

Each ml contains:

Calamine	100.0 mg
Zinc oxide	30.0 mg
Diphenhydramine HCl	10.0 mg
Camphor	15.0 mg
Menthol	5.0 mg
Ethyl alcohol 95%	0.05 ml

APPENDIX E

TEST OF SIGNIFICANCE [47]

In developing a new analytical method, it is often desirable to compare the results of that method with those an accepted or standard method and decide whether where is a statistical difference between the results obtained. The *t* test is very useful for such comparisons. The new method which used for the varying composition is frequently tested against an accepted method by analyzing several different samples. The difference between each of the paired measurements on each sample is computed. An average difference $\overline{D}$ is calculated and the individual deviations of each from $\overline{D}$ are used to compute a standard deviation, s_d. The *t* value is calculated from:

$$t = \frac{\overline{D}}{s_d}\sqrt{N}$$

$$s_d = \sqrt{\frac{\sum(D_i - \overline{D})^2}{N-1}}$$

D_i = The individual differences between the two methods for each sample, with regard to sign.

$\overline{D}$ = The mean of all the individual differences.

Following are two sets of results for a number of individual samples.

Sample type	Proposed method (%L.a)	HPLC (%L.a)	D_i	$D_i - \overline{D}$	$(D_i - \overline{D})^2$
Capsule	101.6	100.0	1.6	7.78	60.52
Syrup1	102.4	100.8	2.0	8.18	66.91
Syrup2	92.0	93.2	-1.2	4.98	24.8
cream	69.8	96.9	-27.1	-20.92	437.65
			$\overline{D}$ = -6.18		$\sum$ = 589.88

$$s_d = \sqrt{\frac{589.88}{4-1}} = 14.02$$

$$t = \frac{-6.18}{14.02} \times \sqrt{4} = 0.88$$

The tabulated t value at the 95% confidence level for 3 degrees of freedom is 3.18. Therefore, $t_{calc} \langle t_{table}$, and there is no significant difference between the two methods at this confidence level.

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