Determination of 15-Acetyldeoxynivalenol and Zearalenone in Corn Oil by ATR-FTIR Spectroscopy

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Determination of 15-Acetyldeoxynivalenol and Zearalenone in Corn Oil by ATR-FTIR Spectroscopy

(ATR-FTIR 分光法によるコーン油中の 15-アセチルデオキシニバレノールおよびゼアラレノンの検出)

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Dedicated to my loving and lovely wife

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ABSTRACT

Determination of 15-Acetyldeoxynivalenol and Zearalenone in Corn Oil by ATR-FTIR Spectroscopy

Mycotoxins are secondary metabolites produced by filamentous fungi. Mycotoxins draw public attention owing to their frequent occurrence in agricultural products and serious toxicities to human beings and domestic animals. Recently, mycotoxin contamination in edible oil was reported around the world. European Commission (EC) has set maximum allowed levels for mycotoxin in cereals and its oil products.

15-acetyldeoxynivalenol (15-AcDON) and zearalenone (ZON) are mycotoxins produced by *Fusarium spp*. 15-AcDON belongs to trichothecene group, which has highly acute toxicity to humans and domestic animals, such as feed refusal and vomiting, while ZON is an estrogenic mycotoxin which could cause breeding problems to human beings. Recently, it has been found that ZON was the most prevalent mycotoxin in edible oil, and 15-AcDON has significantly higher contamination level than the other trichothecene mycotoxins.

Instrumental methods, such as HPLC, GC and HPLC-MS-MS, have been developed for determination of mycotoxin in edible oil. These methods are reliable and have been used in laboratory. However, extraction and purification before instrumental analysis are time-consuming, and the reagents used in these procedures are not friendly for environment. In addition, the instrumentation analysis requires high levels of experience and expertise for operations. Therefore, these methods are not suitable for rapid determination of mycotoxin on site.

In this study, a rapid, non-destructive and easy method was developed for determination of 15-AcDON and ZON in corn oil by ATR-FTIR spectroscopy. In Chapter I of this thesis, the background and objectives of this investigation were introduced as stated above. Chapter II is literature review. In this chapter, the contamination, regulation, determination of mycotoxin in edible oil, and elimination of oil refining process on mycotoxin were stated. Moreover, the principle of ATR-FTIR spectroscopy and its application for determination of mycotoxin in previous studies were presented and the feasibility of ATR-FTIR spectroscopy for determination of mycotoxin in edible oil was discussed.

In Chapter III, characterization of 15-AcDON and ZON in corn oil was investigated by ATR-FTIR spectroscopy combined by PCA. The corn oil samples with 15-AcDON at 0, 0.1, 1, 10 and 100 ppm or with ZON at 0.1, 0.5, 1.2, 2.25 and 10ppm were discriminated according to the relevant mycotoxin concentration in PCA scores. The specific bands of 15-AcDON or ZON were identified in different spectral regions depending on the high and low concentration of 15-AcDON or ZON by PCA loadings. For the high concentrations of the mycotoxins, the specific bands of 15-AcDON were located at 1044-1003 cm^{-1} which corresponded to the C-O of 15-AcDON, and the IR absorption of ZON at 1230-1150 cm^{-1} resulted from the aromatic ring of ZON. For the low concentrations of the mycotoxins, the specific bands of 15-AcDON were at 1090-1075 cm^{-1} and assigned to the interaction between 15-AcDON molecules and C-O of corn oil, while the specific bands of ZON were found at 1760-1730 cm^{-1} and assigned to the interaction between ester C=O of corn oil and ZON molecules. Therefore, the specific bands of 15-AcDON and ZON with high concentration were directly caused by the vibrations of their own functional groups, while the specific bands of 15-AcDON and ZON with low level were caused by the interaction between the mycotoxin and composition of corn oil.

In Chapter IV, 15-AcDON in corn oil was determined by ATR-FTIR spectroscopy with multivariate models. The total 57 corn oil samples were split into 36 as calibration set and 21 as validation set, and the 15-AcDON concentration was prepared at 0.1-2.25ppm. PLS, MLR and PCR models were optimized by using different preprocessing methods and the informative spectral regions selected by GA-PLS, iPLS, GA-MLR, MLR step, and iPCR. By comparison between the results of preprocessing methods, it was found that Detrend(0)-mean center for PLS, Baseline correction–mean center for MLR, and standard normal variate (SNV)-mean center for PCR generated superior cross-validation and prediction results. Comparing with the results of PLS, MLR and PCR models in the full region (4000-600 cm^{-1}), GA-PLS, iPLS, GA-MLR, and iPCR had superior predictability. In addition, GA-PLS and iPLS provided better cross-validation and prediction results, compared to other methods with optimized spectral regions. IPLS had R_{CV}^2 of 0.829, RMSECV of 0.260, and RMSEP of 0.436, R_P^2 of 0.748. Compared with iPLS, GA-PLS had the lower R_{CV}^2(0.789), higher RMSECV (0.291), but higher R_P^2 of (0.841) and lower RMSEP (0.211). Therefore, GA-PLS

demonstrated superior prediction performance to iPLS.

In Chapter V, PLS model was developed for determination of ZON in corn oil. The total 66 corn oil samples were split into 45 as calibration set and 21 as validation set, and ZON concentrations of corn oil were prepared at 0.5-1.9ppm. The spectral regions for PLS were initially selected by PCA, followed by VIP scores, regression vector and iPLS. However, the spectral regions by VIP scores and Regression vector did not improve the model. The iPLS produced better cross validation result, but inferior prediction, compared to the PLS with 1830-883 cm^{-1} region selected by PCA. As a comparison to PLS and iPLS, PCR and iPCR were applied in the 1830-883 cm^{-1} region. Compared to PCR, iPCR produced better cross validation result, but inferior prediction, which was identical to the comparison between PLS and iPLS. The inferior predictions of iPLS and iPCR confirmed that the spectral region of 1830-883cm^{-1} was specific for ZON in corn oil. The optimum PLS model with the spectral range of 1830-883 cm^{-1} had R_p^2 of 0.732, and RMSEP of 0.299.

In Chapter VI, ATR-FTIR spectroscopy was applied to simultaneously determine 15-AcDON and ZON in corn oil. A total of 69 corn oil samples with 15-AcDON at 0.1-2.25ppm and ZON at 0.075-1.9ppm were prepared. All the data were used as calibration for selecting the informative spectral regions. Firstly, the spectral regions were initially selected by PRVS (Preprocessing plot, Regression Vector, VIP Scores and Selectivity Ratio). Then, GA-PLS and iPLS were further applied for the spectral region selected by PRVS. Compared to the PLS models with the full spectral region, the PLS models for 15-AcDON and ZON based on PRVS, GA-PLS and iPLS were found to yield better predictability. Specifically for 15-AcDON, iPLS demonstrated superior cross validation performance to GA-PLS. For ZON, GA-PLS model generated higher improvement to iPLS. The cross validations of the PLS models for 15-AcDON and ZON were evaluated by using 70% of the total spectra as calibration, and 30% of the total spectral data as validation. For 15-AcDON, the optimum prediction by iPLS had R_p^2 of 0.784 and RMSEP of 0.293. For ZON, the PLS model based on GA-PLS showed higher prediction ability with R_p^2 of 0.644 and RMSEP of 0.339, which could be used for discriminating the low from high levels of ZON in corn oil. Furthermore, the simultaneous determination of 15-AcDON and ZON was compared to the separate determination of 15-AcDON or ZON in Chapter IV and V. It was found that both

contaminants have strong interference with each other on the spectral regions and prediction results. For 15-AcDON, the simultaneous determination used window size of 3 variables for iPLS, while Chapter IV used 10 variables in GA-PLS for separately determining 15-AcDON. The smaller window size (3 variables) of the simultaneous determination was reasonable, because the IR bands of 15-AcDON and ZON were overlapped. Compared to the separate determination, the iPLS model of the simultaneous determination showed inferior prediction result, suggesting that ZON has interference on the detection of 15-AcDON. For ZON, compared to the separate determination, the GA-PLS model of the simultaneous determination showed inferior prediction result, suggesting that 15-AcDON has interference on the detection of ZON in corn oil. Moreover, for separate determination of ZON in corn oil, Chapter V found that the region of 1830-883 cm^{-1} produced better prediction results compared to the regions selected by iPLS and GA-PLS. In the simultaneous investigation, GA-PLS with window size of 10 variables generated better results for determining ZON in corn oil with 15-AcDON.

Therefore, ATR-FTIR spectroscopy has the potential for characterization of the IR band variation with the concentration of 15-AcDON. And it is feasible for separately and simultaneously determining 15-AcDON and ZON in corn oil by using ATR-FTIR spectroscopy.

Keywords: ATR-FTIR spectroscopy, 15-AcDON, ZON, principal component analysis (PCA), partial least squares (PLS), principal component regression (PCR), multiple linear regression (MLR), genetic algorithms (GA), interval partial least squares (iPLS), interval principal component regression (iPCR), variable importance in projection (VIP) scores, mycotoxin

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LIST OF ABBREVIATIONS

ATR	attenuated total reflectance
FTIR	Fourier transform infrared
GA	genetic algorithms
HPLC	high performance liquid chromatography
GC	gas chromatography
IR	infrared
MS	mass
ZON	zearalenone
DON	deoxynivalenol
15-AcDON	15-acetyldeoxynivalenol
Tri	trichothecenes
AFs	aflatoxins
OTA	ochratoxin A
PLS	partial least squares
PCA	principal component analysis
PCR	principal component regression
MLR	multiple linear regression
iPLS	interval partial least squares
iPCR	interval principal component regression
R^2	coefficient of determination
RMSEC	root mean square of calibration
RMSECV	root mean square of cross validation
RMSECP	root mean square of prediction
LV	latent variables
PRVS	Preprocessing plot, Regression Vector, VIP Scores and Selectivity Ratio
ppm	Parts per million (equals μg/ml)
ppb	Parts per billion (equals μg/L)

CHAPTER I

INTRODUCTION

1.1 Introduction

Nowadays, the occurrence of mycotoxins reported in various foodstuffs has seriously threatened the health of human beings, due to the varieties of the toxicities.

15-acetyldeoxynivalenol (15-AcDON) and zearalenone (ZON) are mycotoxins produced by *F*usarium spp. 15-AcDON is acetylated deoxynivalenol (DON) derivative, belonging to trichothecenes group, while ZON is a resorcyclic acid lactone, and a non-steroidal estrogenic mycotoxin (Bennett and Klich, 2003; Zinedine et al., 2007). The characteristic toxicity of 15-AcDON is associated with feed refusal and vomiting, and ZON can cause serious breeding problems, such as infertility, abortion. The contamination of 15-AcDON and ZON occurred in various foodstuffs, mainly in cereals and cereal-based foods. Recently, attention was raised on the contamination of 15-AcDON and ZON in edible oil. It has been found that ZON was the most prevalent mycotoxin in edible oil, and 15-AcDON has significantly higher contamination level than other trichothecenes (Li et al., 2014; Schollenberger et al., 2008). Because of the frequent contamination, high contamination levels, and the varieties of toxicities of 15-AcDON and ZON, regulations of some countries and organizations have been established. European Commission has recently set out the harmonized maximum limits of 0.2-1.75 ppm for DON in cereals and of 0.4 ppm for ZON in refined corn oil (Commission Regulation (EC) No 1881/2006 of 19). JECFA (Joint FAO/WHO Expert Committee on Food Additives) (2010) has set the Provisional Maximum Tolerable Daily Intake (PMTDI) to the group of DON and its acetylated derivatives 3-AcDON and 15-AcDON at 1 μg/kg body weight (b.w.) per day.

Recently, the instrumental methods, such as HPLC, GC, and HPLC-MS/MS, have been applied for determining mycotoxin in edible oil. These methods are precise and accurate, and suitable used in laboratory. However, the extraction and purification of the mycotoxin before instrumental analysis are time-consuming, and the operation of the instrumentation requires a high level of expertise and experience.

Molecular absorption of energy in the near infrared (NIR) region (12,500-4,000

cm^{-1}) results from the excitation of overtones and combinations of the fundamental vibrational modes that give rise to mid-infrared (MIR, 4,000-400 cm^{-1}). The NIR absorption is weaker and generally only observed for bonds involving hydrogen (e.g. C-H, O-H, N-H). Therefore, NIR spectroscopy cannot provide the same kind of detailed structural information as MIR spectroscopy. Moreover, in recent decades, chemometrics have been prevalently applied with IR spectroscopy, such as principal component analysis (PCA), partial least squares (PLS), and so on. Accordingly, NIR and MIR spectroscopy has become a fast and non-destructive alternative to chemical means not only for qualitative characterization but also quantitative measurement of contaminants in food. NIR spectroscopy was used for characterizing and screening DON ranged 0-90 ppm in Fusarium-damaged wheat kernels (Peiris et al., 2009; Dvořáček et al., 2012; Pettersson and Åberg. 2003). Tripathi and Mishra et al. (2009) determined aflatoxin B1 ranged 15-500 ppb in red chili powder by using NIR spectroscopy. In recent years, ATR-FTIR spectroscopy was applied to determine mycotoxin in wheat, corn and groundnut (Abramovic et al., 2007; Kos et al., 2007; Mirghani et al., 2001). Peiris et al. (2012) investigated the MIR bands difference between sound and Fusarium-damaged wheat kernels by ATR-FTIR spectroscopy.

From the previous investigations, it has been demonstrated that NIR and MIR spectroscopy have been applied for characterizing and determining the mycotoxins in solid samples, such as cereals, groundnut, and chili. Nonetheless, so far no investigation was made to evaluate mycotoxin in edible oil. According to the previous research, it is possible to characterize and predict mycotoxin in edible oil by ATR-FTIR spectroscopy. Moreover, the PLS models for prediction of DON in wheat and corn used the spectral region ranged 1800-800 cm^{-1}. Although PLS model has built-in ability to overcome the over-determined problem of full spectral calibration, the selections of preprocessing methods and spectral regions are still important (Du et al., 2004; Luypaert et al., 2004). It is useful to select a spectral region and a suitable preprocessing method that improves the prediction accuracy and interprets the PLS results (Du et al., 2004; Luypaert et al., 2004). Therefore, it is necessary to find the proper preprocessing methods and spectral regions to develop the robust models of 15-AcDON and ZON in edible oil by using ATR-FTIR spectroscopy.

Besides, it has been found that several mycotoxins simultaneously occurred in

edible oil in China (Li et al., 2014). Nowadays, the simultaneous determination of several mycotoxins was performed usually by using GC-MS and HPLC-MS/MS (Siegel et al., 2010; Gentili et al., 2007; Berthiller et al., 2007), but these instrumental methods are time-consuming and labor intensive, and not suitable for rapid determination. So far, the simultaneous determination of ingredients or pesticides has been developed by the use of ATR-FTIR spectroscopy (Ahmadi & Arshadi, 1999). However, few studies have been made to develop a rapid method to simultaneously quantify several mycotoxins by MIR or NIR spectroscopy. Therefore, it is necessary to investigate the feasibility of ATR-FTIR spectroscopy for rapidly simultaneous determination of several mycotoxins.

In this study, 15-AcDON or ZON contaminated corn oil with several concentration levels were firstly characterized by ATR-FTIR spectroscopy. The specific IR bands of 15-AcDON and ZON were identified and the contaminated corn oil samples were discriminated according to the concentration level by using principal component analysis (PCA). Secondly, in order to compare and improve the prediction results, several multivariate models with different spectral region selection and preprocessing methods were applied for determination of 15-AcDON and ZON in corn oil. For determination of 15-AcDON, the multivariate models of partial least squares (PLS), multiple linear regression (MLR) and principal component regression (PCR) were employed, and interval PLS(iPLS), genetic algorithm-PLS (GA-PLS), interval PCR (iPCR) and GA-MLR were applied for selecting suitable IR regions to improve the performance of the models. For determination of ZON, the multivariate models PLS and PCR were used, and PCA, Variable importance in projection (VIP), iPLS and iPCR were applied for selecting suitable IR region. In addition, the 15-AcDON and ZON in corn oil was simultaneously determined by ATR-FTIR spectroscopy. GA-PLS and iPLS were used for generating suitable wavenumber region for prediction of 15-AcDON and ZON. The separate and simultaneous determination of 15-AcDON and ZON was compared.

1.2 Objectives

The aim of this study was to develop a rapid and non-destructive method for determining 15-AcDON and ZON in corn oil by ATR-FTIR spectroscopy. Specifically, the objectives were as follows:

a) To identify the specific bands of 15-AcDON and ZON and to discriminate 15-AcDON or ZON in different concentration levels by ATR-FTIR spectroscopy combined by PCA.

b) To investigate the feasibility of the application ATR-FTIR spectroscopy for separately determining 15-AcDON and ZON in corn oil.

c) To investigate the potential for applying ATR-FTIR spectroscopy for simultaneously determining 15-AcDON and ZON in corn oil.

CHAPTER II
LITERATURE REVIEW

2.1 Mycotoxin contamination in edible oil

Edible oil is one of the most required vegetable products worldwide. Due to its wide application in several fields, edible oil has been used in various products for human practicality and comfort (Murphy et al., 1993). Edible oils were found in natural products where levels of contaminants may vary with the type of raw materials, in addition to external factors such as climate, harvesting, processing conditions and storage (Banu & Muthumary. 2010; Davie & Vincent. 1980). Raw material for the production of edible oils often store for weeks before the process of production of edible oil, where the molds and mycotoxins can be increased.

Mycotoxins are toxic metabolites produced by fungi. The predominant mycotoxins in edible oil are aflatoxins(AFs), trichothcenes(Tri), zearalenone(ZON) and *Alternaria* toxins. These toxic compounds pose a potential threat to human and domestic animal health through the ingestion of food products. (Peraica et al., 1999; Richard, 2007).

Wide concern on the mycotoxin contamination in edible oil has been raised due to the high contamination of oilseeds and their oil products. The occurrence of mycotoxin in edible oils has been reported around the world, as can be seen in Table 2.1.

In Asia, the most frequently reported mycotoxins are AFs. For crude oil, the levels of AFs in Sri Lanka coconut oil (Samarajewa 1983, Samarajewa et al., 1983), and Indian sunflower (Banu and Muthumary, 2010) and rice bran oils (Jayaraman and Kalyanasundaram, 2009) are worth noting. For refined oil, in China, high levels of aflatoxins were detected in peanut oil from 3 different brands collected in Guangdong Province, creating a concern for local public health (Asian business daily, 2012). Besides that, Li et al (2014) reported that ZON was the most prevalent toxin in China, with the incidence of 27.6% (range=10.0-440.0ppb), and the concentration of DON was ranged 100-700 ppb. The simultaneous contamination with several mycotoxins was also found in the oil products in this report.

In Africa, AFs contamination received the most attention. Aflatoxin B1 was found in over 85% of peanut oil samples with mean contents around 40 ppb (Muleta et al., 2001).

However, the contamination level in Sudanese edible oils was relatively lower. Idris et al.(2010) compared the contamination of AFs in refined and unrefined oil, and found that aflatoxin B1 of unrefined sesame and groundnut oil ranged 0.2-0.8 ppb, and 0.6 ppb, respectively, but no AFs was found in refined oil.

Due to the great production and consumption of olive oil products, European countries paid great attention on mycotoxins contamination in olive oil. Among these countries, Italy did more jobs on the determination of AFs, ochratoxins, and *Alternaria* toxins in olive oils (Cavaliere et al., 2007, Ferracane et al., 2007, Visconti et al., 1986). In addition, attention was also paid on the Fusarium toxins, such as Trichothecenes and ZON. Kappenstein et al., (2005) investigated ZON of the total 77 edible oil samples in Germany, and found the wheat germ oil samples were positive for ZON with the mean concentration of 170ppb. In 2008, Schollenberger et al. (2008) also surveyed the presence of ZON in edible oils. ZON was found in 12 samples (n = 110), and the mean and maximum ZON concentrations were 385 and 1730ppb, respectively. A very high contamination rate (86.4 %) with ZON level up to 823ppb was found in corn oil and wheat germ oil. Mean concentration in corn oil (mean = 90ppb) was about 3 times higher compared to wheat germ oil (mean = 31ppb). Due to the high contamination level of ZON, especially in corn oil and wheat germ oil, these food groups made a striking contribution to the exposure. Although the amount of edible oil consumed is smaller compared to cereals, in some dietary studies it accounted for more than 50 % of ZON exposure (EFSA, 2011).

Table 2.1 Occurrence of mycotoxins in edible oils

Zone	Country	Oil type	Mycotoxin	Positive sample(%)	Levels(ppb)	Reference
Asia	China	cereal and oil products	AFB1	14.5	1.0-32.2	Li, 2014
			ZON	27.6	10-440	
			AFs	40	1.1-35	
			DON	5.9	100-700	
			OTA	14.5	0.51-16.2	
		Peanut oil	AFs	76.6	Minimum:8000	Mehan and Gowda, 1997.
		vegetable oils	AFs	3.7	Range 0.1-5.8	HKSAR, 2001.
		Peanut oil	AFs	66	Range 100-52500	Siame, and Nawa, 2008.
		Sesame oil	AFs	NS	Maximum:20500	Li et al., 2009.
	India	Mustard oil	AFs	33	55-87	Sahay and Prasad, 1990.
		Rice bran crude oil	AFs	75	NS	Jayaraman and Kalyanasundaram, 2009.
		Rice bran refined oil		30	Up to 956; average: 618	
		sunflower	AFB1	10/23	40-600	Banu and Muthumary, 2010.
	Japan	Peanut oil	AFs	62.5	0.01-0.72	Kamimura, et al., 1986.
	Sri Lanka	Coconut oil	AFs	57	Up to 2, average: 186	Samarajeewa et al., 1983.
	Sri Lanka	Coconut oil	AFs	NS	Average:50	Samarajewa. 1983.
Africa	Sudan	Sesame, peanut and sunflower oils	AFs	98.8	Range:0.4-339.9; Average: 57.5	Elzupir et al., 2010.
		Peanut oil	AFB1	85	Mean: 40	Muleta and Ashenafi, 2001
		unrefined sesame and	AFs	NS	0.2-0.8	Idris, 2010.

		groundnut oil				
Europe	Italy	Olive oil	OTA	80	Up to 17	Ferracane et al., 2007
	Spain	Wheat germ oil	DON	40	41	Giménez et al., 2013.
	Spain	Wheat germ oil	ZON	16	6	
	Germany	Edible oil	Tri	NS	Not exceed 116	Schollenberger et al., 2008.
		Edible oil	ZON	NS	Up to 1730	
		Edible oil	ZON	86.4	Up to 823	Kappenstein et al. 2005
	Spain	Refined corn oil	ZON	32	0-24	Escobar et al. 2013.
		Refined corn oil	DON	NS	106, 216	
	Italy	Olive oil	ALT	NS	AME:793; AOH:289	Visconti et al., 1986.

AFs: aflatoxins AFB1: aflatoxin B1; Tri: trichothecenes; OTA: ochratoxin A; ALT: alternaria toxins; AME: Alternariol Methyl Ether; AOH: Alternariol; NS: not specified;

2.2 Regulations on mycotoxins contamination

Maximum allowed limit for mycotoxins has been established in several organizations and countries, with specifications for different foodstuffs. For AFs, the EC has set the maximum limited level of 15ppb in oilseeds, and USA of 20ppb in foods except milk and India of in grain and peanut products. The maximum allowed levels in African countries ranged 4-30 ppb. For DON, the maximum limited level by EC was at 750ppb for cereal products, while China at 1000ppb for corn and corn products. In addition, JECFA (2001, 2010) set the Provisional Maximum Tolerable Daily Intake (PMTDI) to the group of DON and its acetylated derivatives 3-AcDON and 15-AcDON at 1 μg/kg body weight (b.w.) per day.

Considering the prevalent contamination of ZON in corn products, EC, China and Japan have set the maximum limits for cereals and/or cereals products. Specifically, EC set the maximum limit of 400ppb for refined corn oil.

Table 2.2 Regulations for mycotoxins in food

Organization/Country	Food	Mycotoxin	Maximum allowed limit (ppb)	Reference
EC	Oilseeds	AFs	15	Commission
	Cereal foods	OTA	3	Regulation (EC)
	Cereals for direct human consumption	DON	750	No 1881/2006
	Refined corn oil	ZON	400	
USA	Foods except milk	AFs	20	FDA, 2004.
	Finished wheat products	DON	1000	Kubo M., 2012.
China	Corn and peanut products	AFB1	20	Kubo M., 2012.
	Corn and corn products	DON	1000	
	Grain and bean	OTA	5	
	Wheat and corn	ZON	60	
Japan	wheat	DON	1100	Kubo M., 2012.
	Compound feed	ZON	1000	FAO,1996
Africa	Foods	AFs	4-30	
India	Grains peanut	AFs	30	
JECFA	For DON, 3-AcDON and 15-AcDON, Provisional Maximum Tolerable Daily Intake (PMTDI): 1 μg/kg body weight (b.w.) per day			JECFA, 2001, JECFA, 2010

2.3 Mycotoxin elimination during oil extraction, purification and refining process

Although studies have shown that mycotoxin of the oleaginous material can be transferred to the oil product, these contaminants could be reduced, depending on the extraction, purification of the crude oil, and the later process of refining (degumming, deacidification, bleaching and deodorization).

For extracting oil from the oleaginous materials, mechanical pressing and solvents, as well as a combination of both were most used according to the characteristics of the materials. Once the raw material contaminated with fungi was used in both mechanical and solvent extraction of oil, the mycotoxins will present in both oil and cake parts. The

extraction can partly remove these contaminants by physical, chemical, thermal and/or microbiological treatments.

However, the oil extraction step was not capable to eliminate all the mycotoxin, and it was found that the mycotoxin remained in both oil and defatted cake. A study conducted by Basappa and Sreenivasamurthy (1974) presented that 85 % of aflatoxins in peanuts remained in cake after extraction and 15 % could be transferred into the crude oil.

After extraction, the crude oil may contain some impurities, such as phospholipids, pigments, waxes and trace metals. These impurities can be harmful to quality of product, being necessary to be removed through the oil refining process (Anderson, 2005). Figure 2.1 provides a diagram description of the chemical and physical refining processes, most used in industry.

Most of the mycotoxins are polar compounds and therefore are not usually found in edible oils (non-polar). However, in crude oils, toxins have presented certain contamination risk (Shephard et al., 2011). Parker and Melnick (1966) were the first researchers to report that the refining process was effective in removing mycotoxins. The authors evaluated the effect of chemical refining on peanut crude oil with afaltoxins of 812 ppb. After the bleaching stage, aflatoxins was found lower than 1ppb in the final oil samples. Kamimura et al (1986) analyzed the effect of chemical refining process on the elimination of mycotoxin (aflatoxins, trichothecenes (Tri) and ZON) of 8 types oils (corn, peanuts, cotton, saffron, olive oil, nuts and sesame seed). Before refining process, crude corn oils were contaminated with aflatoxins at 0.8-1 ppm, DON at 8 ppm, nivalenol at 8ppm and ZON at 10 ppm. After refining process, the mycotoxins were not found in the obtained refined oil. In fact, alkaline neutralization, washing, bleaching and deodorization of the refining process could remove the mycotoxins present in the previous samples (Kamimura et al., 1986).

Mycotoxins can be partially destroyed by the conditions of each refining stages. Although degumming is not mandatory for the oil chemical refining, the main reasons for performing this step are to make the oil suitable for storage and physical refining (Anderson, 2005). In this step, crude oil is treated with water or diluted acid in order to hydrate the phosphatide (Pagliero et al., 2001). There are no studies in the literature describe the relationship between degumming and mycotoxins. However, it is known

that mycotoxins are low-polar and soluble in polar solvents (Ferracane et al., 2007). Thus, it is assumed that this step may reduce contamination of the vegetable oil.

In the deacidification step, the removal of free fatty acids (deacidification) was conducted by using alkali(chemical refining) or water vapor under high temperature and low pressure (physical refining) (Figure 2.1). The alkali treatment is effective for degrading mycotoxins by hydrolysis of the lactone or ester bond in some mycotoxin molecules, such as AFs, ZON, and some Tri (Park, 2002). Kamimura et al.(1986) found that after treatment with sodium hydroxide, aflatoxins B1 and B2 reduced more than half of the initial level in the first 2 min. However, ZON was not reduced significantly and the Tri were reduced by 50 % in 8 min.

In the bleaching step, a treatment of solid bleaching agent (bleaching earth or clay) was applied under vacuum conditions and high temperature, and the agent removal by filtration (Anderson 2005), which results in the oil less colorful and more stable. The study by Kamimura et al. (1986) evaluated the effect of activated clay (2 % to the oil by weight) for eliminating mycotoxins in oil. The authors noticed that Tri and aflatoxins were removed of after 15 min of interaction. However, even in 30 min contact, 82 % of the initial amount of ZON was still left in the bleached oil.

The last stage of the refining process is deodorization, which is a desorption process including high temperature (220-270 ℃) and low pressure (1-5 mmHg) for eliminating the impurities, such as aldehydes and ketones (Davie and Vincent, 1980). Toxicity of AFs, Tri, ZON and fumonisins could be reduced at temperatures greater than 150 ℃ (Bullerman and Bianchini, 2007). Kamimura et al. (1986) evaluated the deodorization process with temperature of 240 ℃, and pressure from 2 to 5 mmHg in artificially contaminated oil with mycotoxins. The authors found that concentration of the toxin was decreased over time, and after 120 min the survival percent was: aflatoxins B1 86 %, B2 80 %, ZON 45 % and Tri 7 %. After 150 min of treatment, Tri were completely eliminated.

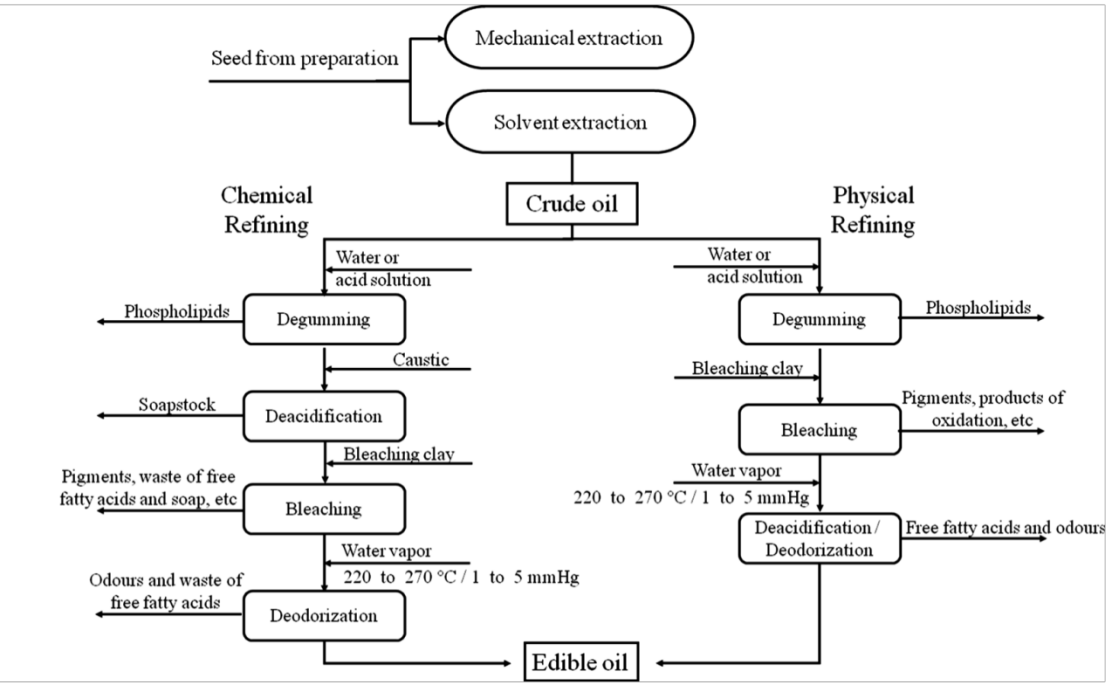

Figure 2.1 Process flow diagram for edible oil production

According to the discussion above, mycotoxin of edible oil could be partly or fully eliminated during the oil refining process. In fact, the elimination efficiency of mycotoxins in refined oil depends on the types of mycotoxin, the refining process (time, temperature, types of refining, etc.), and the concentration levels of mycotoxins in crude oil. Moreover, the mycotoxin contamination found in refined oil suggested that it is necessary to develop the methods for determining mycotoxin in edible oil.

2.4 Detection of mycotoxin in edible oil by instrumental methods

The methods for determining mycotoxin contamination in edible oil are generally high performance liquid chromatography (HPLC) (Miller et al, 1985; Siegel et al., 2010; Visconti et al., 1986), HPLC-MS-MS (Peng, et al., 2009; Gentili et al., 2007) and thin layer chromatography (TLC) (Hagan and Tietjen, 1975; Visconti et al., 1986). The analytical methods for the determination of mycotoxin in food generally consist of extraction, cleanup, quantification and validation. Extraction of mycotoxin from oil samples requires solvents or mixtures of water and polar solvents, and hexane.

The extract resulting from the previous step contains various impurities such as lipids and pigments. Cleanup procedures involve liquid–liquid partition, solid phase

extraction and immune-affinity columns (Bao et al., 2013; Krska et al., 2005). Despite the rapid advance in methods for detection and quantification of mycotoxins, the conventional HPLC and LC-MS/MS methods are the most widely used (Ferracane et al., 2007; Bao et al., 2010; Mahoney and Molyneux, 2010; Gentili et al., 2007; Boonzaaijer et al., 2008; Yang et al., 2011). The methods of analysis for mycotoxins in oils provided reproducibility, accuracy, precision. However, the extraction and purification procedures are time-consuming, and the detections require high levels of experience and expertise.

2.5 Infrared (IR) Spectroscopy

2.5.1 Principles of IR spectroscopy

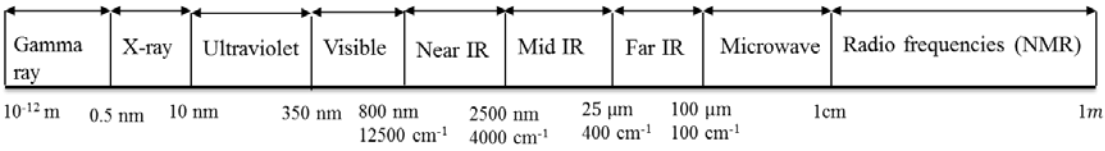

Figure 2.2 The region of the electromagnetic spectrum

The infrared region of the electromagnetic spectrum extends from 12500-100 cm^{-1}. The mid-infrared region (MIR) extends from 4000 cm^{-1} to 400 cm^{-1}. It is surrounded by the far-IR region (FIR) from 400 cm^{-1} to 100 cm^{-1} and the very important near-IR region (NIR) from 12500 cm^{-1} to 4000 cm^{-1} (Figure 2.2) (Gauglitz and Vo-Dinh et al., 2006).

Infrared spectroscopy is a technique based on the vibrations of the atoms of a molecule. IR radiation does not have enough energy to induce electronic transitions as seen with UV. Absorption of IR is restricted to compounds with small energy differences in the possible vibrational and rotational states. For a molecule to absorb IR, the vibrations or rotations within a molecule must cause a net change in the dipole moment of the molecule. The alternating electrical field of the radiation interacts with fluctuations in the dipole moment of the molecule. If the frequency of the radiation matches the vibrational frequency of the molecule, then radiation will be absorbed, causing a change in the amplitude of molecular vibration. In fact, IR spectroscopy is primarily concerned with molecular vibrations, since transitions between individual rotational states can be measured only in the IR spectra of small molecules in the gas phase.

A non-linear molecule compose of n atoms has 3n-6 degrees of freedom corresponding to vibrational motions, and the molecule is said to possess 3n-6

vibrational modes. In addition, a linear molecule has 3n-5 normal vibrational modes. These vibrational modes are described as bond stretching vibrations and different types of bending vibrations (Figure 2.3).

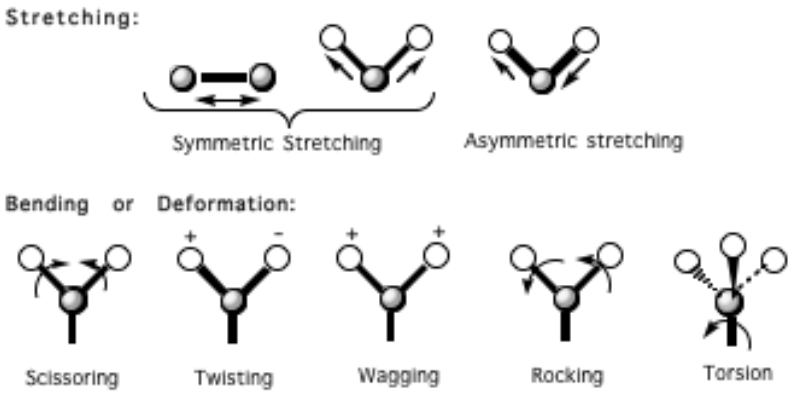

Figure 2.3 Types of molecular vibrations: stretching and bending

An IR absorption spectrum can be produced by collecting the radiation after its interaction with the material. Fundamental vibrations, which occur in the mid-IR region (4000–600 cm^{-1}), are commonly studied in spectral IR analysis. MIR vibration has strong water absorption band but the effect can be overcome by subtraction. The MIR spectrum has well-resolved peaks whereas broad overlapping absorption bands in the NIR spectrum make structural selectivity and band assignment to a specific chemical compound very difficult (Smith, 2011).

MIR spectroscopy has been commonly used for the structural identification or qualitative determination of the “fingerprint” of organic compounds, because some functional groups display characteristic vibrational absorption frequencies in this infrared region. Moreover, MIR spectroscopy is amendable to quantitative analysis application, because the absorptions of the bands in a spectrum are proportional to the concentrations of their respective functional groups according to Lamber-Beer’s law (equation 2.1). Where, A is the absorbance of a band, b is the path length cell in MIR measurement, ϵ is a molar proportionality constant called molar absorption which is the characteristic of each functional group, and c is the concentration of the functional group.

$$A = \epsilon bc \qquad (2.1)$$

The study of food quality may strongly profit from MIR spectroscopy, in that whether components or contaminants, or the interaction between components and contaminants of foods could show characteristic absorption bands in this region of the electromagnetic spectrum. Therefore, in recent years, MIR has been of increasing interest in food quality control. And with the developments of Fourier transform infrared (FTIR) spectroscopic technique and attenuated total reflectance (ATR) as a simple sample handing technique, MIR spectroscopy has a substantial potential to become a quantitative quality control for food industry.

2.5.2 Fourier transform infrared spectroscopy (FTIR)

The term Fourier transformation has originated from a mathematical operation demonstrated by ‘Jean Fourier’ which converts the frequency domain into time domain. In 1949, astrophysicist Peter Fellgett used an interferometer to measure light from celestial bodies and produced the first Fourier transform infrared spectrum. However, because of the requirement of large, expensive computers with 12 hours to transform an interferogram into a spectrum, FTIR spectrometers were limited to dispersive techniques for investigations (Sheppard, 2006). In the late 1960s, commercial FTIR spectrometers appeared with the advance of microcomputers available for Fourier transform (Sun, 2009).

Fourier transform infrared (FTIR) spectrometers have almost entirely replaced dispersive instruments because of their better performance in terms of speed and efficiency. The optical system in FTIR spectrometer is very simple: the interferometer requires two mirrors, an infrared light source, an infrared detector, and a beam splitter (Hsieh, 2008).

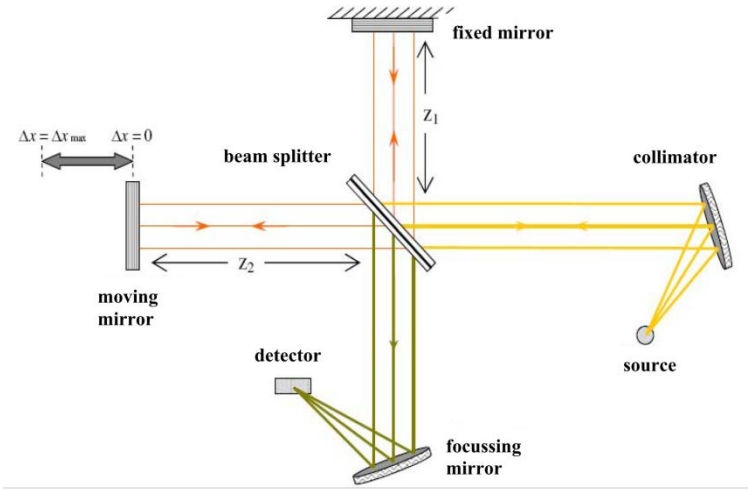

Figure 2.4 The Michelson interferometer

The beam splitter is the heart of the interferometer. The beam splitter reflects about half of an incident light beam while simultaneously transmitting the remaining half. One half of this split light beam travels to the interferometer's moving mirror while the other half travels to the interferometer's stationary mirror. The two mirrors reflect both beams back to the beam splitter where each of the two beams is again half reflected and half transmitted. Then, one output beam travels to the detector as the other travels to the source. When the two beams return to the beam splitter, an interferogram is generated. The interferogram, detected by the infrared detector as variations in the infrared energy level, ultimately yields spectral information (Hsieh, 2008). After that, the data are converted to the frequency with a fast Fourier transform (FFT) algorithm. And this plot of spectral data vs. frequency, so called reference or background spectrum, to obtain the transmittance (*T*) spectrum of a sample. Generally, the transmittance spectrum is mathematically transformed to an absorbance spectrum according to the equation 2.2

$$\mathrm{A} = \log(1/T) \qquad (2.2)$$

2.5.3 Attenuated total reflectance (ATR)

Transmission is the most basic technique where infrared irradiation is passed through the sample and the transmitted radiation is measured. This method is useful for thin samples (<10 μm), or during the investigation of weak bands like overtones in thicker samples. Solid samples may require preparation before the measurement for

example preparing KBr pellets, which is a time consuming process and difficult to reproduce.

Attenuated total reflectance (ATR) spectroscopy is based on the principle of total internal reflection. A beam of radiation entering a crystal will undergo total internal reflection when the angle of incidence at the interface between the sample and crystal is greater than the critical angle, where the latter is a function of the refractive indices of the two surfaces. In ATR, the sample is placed in contact with an ATR crystal, which is composed of a material with a high index of refraction, such as zinc selenide (ZnSe). The IR beam is focused onto the beveled edge of the ATR element by a set of mirrors, reflected through the crystal, and then directed to the detector by another set of mirrors. The beam penetrates a fraction of a wavelength beyond the reflecting surface. When a material that selectively absorbs radiation is in close contact with the reflecting surface, the beam loses energy at the wavelength where the material absorbs. The resultant attenuated radiation is measured and plotted as a function of wavelength by the spectrometer and gives rise to the absorption spectral characteristics of the sample (Stuart, 2005). The depth of penetration d_p is determined by equation 2.3.

$$d_p = \frac{\lambda}{2\pi n_1 \left[sin^2(\theta) - \left(\frac{n_1}{n_2}\right)^{\frac{1}{2}} \right]} \qquad (2.3)$$

Where λ is the wavelength of the radiation in ATR crystal, n_1 is the refractive index of the crystal, n_2 is the refractive index of the sample, and θ is the angle of incidence.

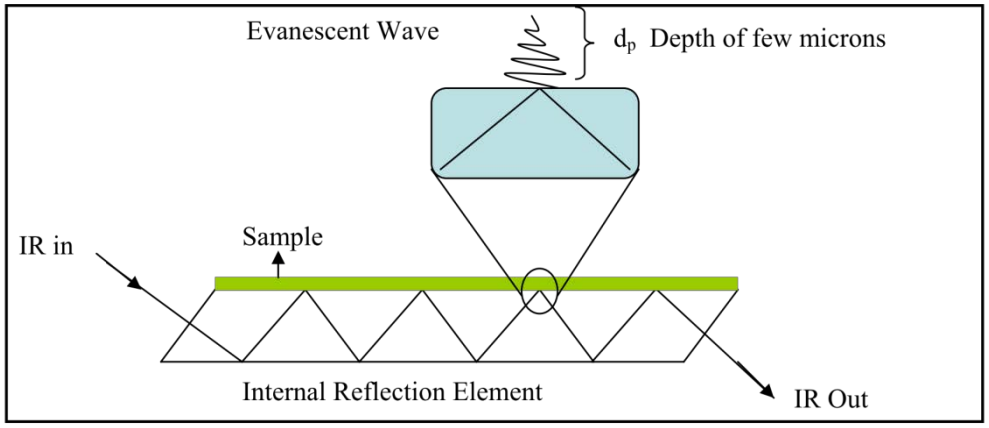

Figure 2.5 Schematic representation of ATR spectroscopy.

ATR is a quick and non-destructive sampling technique for obtaining the IR spectrum of a material's surface. Samples examined by FTIR-ATR generally require minimum, or no, sample preparation.

2.5.4 ATR-FTIR for mycotoxin determination

In recent decades, some studies have found that it is possible to extract the specific information caused by mycotoxins by ATR-FTIR spectra and preprocessing methods. Specifically, band shift and the difference of absorption intensity could be identified in the ATR-FTIR spectra using second derivative algorithm and subtraction method (Peiris et al., 2012; Mirghani et al., 2001). Moreover, ATR-FTIR spectra in combination with multivariate statistical techniques make it feasible for qualitatively and quantitatively determining the mycotoxins in various foods. For the qualitative analysis, classification methods, such as PCA, cluster analysis (CA) make it possible to discriminate the contaminated and non-contaminated samples, and to separate the contaminated samples with different contamination levels of mycotoxin (Kos et al., 2004; Abramovic et al., 2007; Galvis-Sánchez et al., 2007). For the quantitative analysis, by the use of ATR-FTIR spectra with PLS, it has been suggested that spectral region from 1800 to 800 cm^{-1} was used to determine DON in corn and wheat, because the intensity of the OH-stretching vibration at ~3300 cm^{-1} had a rather large variation by moisture, and The C-H stretching bands of CH_2 group at 2925 and 2855 cm^{-1} are not specific enough. The range between 1800 and 2340 cm^{-1} was removed, because it represented absorptions from the internal reflection element (Kos et al., 2004; Abramovic et al., 2007). From the findings of some investigations, it was indicated that the accurate quantification of low DON, OTA and AFs concentrations (ppb level) can be resolved (Kos et al., 2004; Abramovic et al., 2007; Galvis-Sánchez et al., 2007; Mirghani et al., 2001). In addition, by using the absorbance ratio of 1709/1743, multiple linear regression (MLR) was also available for quantification analysis of DON (Abramovic et al., 2007).

To sum up, FTIR spectroscopy has the possibility for rapid, easy, low-cost quantitative analysis of mycotoxin in foodstuffs.

CHAPTER III

APPLICATION OF ATR-FTIR SPECTROSCOPY AND PRINCIPAL COMPONENT ANALYSIS IN CHARACTERIZATION OF 15-ACETYLDEOXYNIVALENOL AND ZEARALENONE IN CORN OIL

3.1 Introduction

15-Acetyldeoxynivalenol (15-AcDON) and Zearalenone (ZON), mycotoxins produced by *Fusarium* spp, are natural contaminants of corn and its oil products in Europe and Asia (Kappenstein et al., 2005; Schollenberger et al., 2008; Li et al., 2014). 15-AcDON, together with deoxynivalenol (DON) and 3-acetyldeoxynivalenol (3-AcDON), is major mycotoxin of the trichothecene group. 15-AcDON raises attention on account of highly acute toxicity and cytotoxicity to humans and domestic animals, while ZON is a mycotoxin with very high estrogenic activity (Bennett et al., 2007; Kuiper et al., 1998; Bryden, 2007). The European Commission has recently set the harmonized maximum limits of 0.2-1.75 ppm for DON in cereals and of 0.1-0.4ppm for ZON in different food products including refined corn oil (Commission Regulation (EC) No 1881/2006 of 19). JECFA (2001, 2010) set the Provisional Maximum Tolerable Daily Intake (PMTDI) to the group of DON and its acetylated derivatives 3-AcDON and 15-AcDON at 1 µg/kg body weight (b.w.) per day.

Incidence and contamination level of Trichothecenes and ZON in edible oil varies depending on the contamination levels in the oleaginous materials and refining methods (physical and chemical). Contamination levels of mycotoxin could be partly eliminated during extraction, purification and refining process. Extraction and purification could remove part of mycotoxin, because most of mycotoxins were soluble in polar organic solvents of extraction and purification process. And refining process could eliminate mycotoxin, because the high temperature, alkali and acidity treatments of oil refining process could destroy the toxicity structures of mycotoxins (Kamimura et al., 1986). On the other hand, ZON contamination is generally more frequent and prevalent than trichothecenes and other mycotoxin groups. In China, ZON was the most prevalent toxin, with the incidence of 27.6% (range= 10.0-440.0 ppb), and the concentration of DON was ranged 100-700 ppb (Li et al., 2014). In addition, it was found that 15-AcDON

had obviously higher contamination levels than other trichothecenes (Schollenberger et al., 2008).

Various instrumental methods have been developed for trichothecenes and ZON in edible oil, including HPLC, GC, HPLC-MS-MS, and TLC. In 1980s and 1990s, TLC was frequently applied for determination of aflatoxins, OTA, and Alternaria toxins in edible oil (Hagan and Tietjen, 1975; Visconti et al., 1986). More recently, HPLC, GC and HPLC-MS-MS have been developed for the mycotoxins analysis (Miller et al, 1985; Siegel et al., 2010; Peng, et al., 2009; Gentili et al., 2007; Schollenberger et al., 2008). TLC was less accurate and precise and could only be used as qualitative or half-quantitative methods. HPLC, GC and HPLC-MS-MS were more reliable used in laboratory. However, extraction and purification procedures in these methods need several hours, and the reagents used in these procedures are not friendly for environment. In addition, the instrumentation analysis requires high levels of experience and expertise for operations.

Because of the advantages of rapidity, little labor and friendly to environment, IR spectroscopy, such as Mid-infrared, and Near-infrared spectroscopy, has been applied in screening and determination of mycotoxin in grains, fruits and spices. The specific bands of mycotoxins were helpful for the mycotoxin characterization and determination by IR/NIR spectroscopy. Recently it was found that the assignments of these bands were related to the mycotoxin concentration. For the high concentration levels (10-1000ppm), the bands assignment of the determination was based on the variation of the mycotoxin concentration. Peiris et al (2012) assigned the IR band shift and intensity changes of wheat kernels at 1160, 1203, 1303 and 1375 cm^{-1} to DON at 0-1000 ppm. Peiris (2009) found the specific NIR bands of DON were in the regions of 1300-1500 nm, and 1700-2000 nm, which was confirmed by Dvořáček et al. (2012) who used 1390-1770 nm and 1880-2070 nm to determine DON of 0-90ppm in wheat kernel. For the low levels of mycotoxin (0-3 ppm), researchers often assigned the specific bands to the variation of the principal constituents caused by the mycotoxin content (protein, lipids, starch), rather than the direct variation of mycotoxin concentration (Tripathi et al., 2009; Abramovic et al., 2009). Therefore, the IR/NIR bands of higher and lower levels of mycotoxin might be located in different regions.

Accordingly, this study characterized the contamination of 15-AcDON and ZON in corn oil by ATR-FTIR spectroscopy. The objectives of this study are

(1). to identify the specific bands of 15-AcDON and ZON with higher and lower concentration levels, and compare the IR band difference between two levels for 15-AcDON and ZON in corn oil.

(2). to discriminate corn oil contaminated with 15-AcDON or ZON in different concentration levels.

3.2 Materials and methods

3.2.1 Sample preparation

15-AcDON standard and corn oil were purchased from Wako Pure Chemical Industries, Ltd. Japan. ZON was purchased from Sigma-Aldrich (Sigma Chemical, St. Louis, M.O.U.S.A.).

Stock solution of 15-AcDON and ZON: 15-AcDON or ZON in pure crystalline form was dissolved in pure methanol to prepare 100ppm 15-AcDON-methanol or ZON-methanol solution.

Artificially 15-AcDON-contaminated or ZON-contaminated corn oil was prepared by spiking the resulting 15-AcDON-methanol or ZON-methanol solution into corn oil, followed by removing the residual methanol of corn oil in a desiccator with reduced pressure.

For 15-AcDON contaminated corn oil, the concentration of 15-AcDON was prepared at levels of 0, 0.1, 1, 10 and 100ppm in 6 replicates.

For ZON-contaminated corn oil, the concentration of ZON was prepared at levels of 0.1, 0.5, 1.2, 2.25 and 10ppm with 3 replicates.

3.2.2 FTIR spectroscopy measurement

IR Spectra were collected using MB3000 FT-IR spectrometer (ABB Inc. Canada) with a MIRacle ZnSe45° attenuated total reflectance (ATR) accessory (PIKE Tech. USA) in the 4000-600 cm^{-1} region. The absorbance of samples was measured at a resolution of 4 cm^{-1} and with 32 scans. Air-conditioner in the laboratory turned on to keep a stable room temperature of 25℃ and a steady level of humidity.

For the measurement of 15-AcDON and ZON standard, all 25ul 100ppm 15-AcDON or ZON methanol solution was pipetted onto a stainless steel tape. This procedure was repeated for 3 times to get 3 replicates of relevant samples. After vaporization of methanol, the residues on the tapes were measured every 30 minutes with the ATR-FTIR spectrometer to confirm the complete evaporation of methanol. The spectra were rationed against a background of stainless steel tape at room temperature.

For 15-AcDON/ZON contaminated corn oil, the absorbance spectrum was collected against a background obtained with a dry and empty ATR cell. Before recording each spectrum, the ATR crystal was cleaned with a KimWipe tissue (Kimberly-Clark Corporation, USA) and 70% ethanol solution to minimize sampling error, the mean of the three spectra were collected from the same corn oil sample for the following analysis step.

3.2.3 Analysis of spectra

Spectral analysis was performed using PLS Toolbox 6.7.1 (Eigenvector Technologies, Wenatchee, WA, USA) running in a Matlab environment (v.7.5 R2007b, Mathworks, USA).

Principle Component Analysis (PCA) was performed on the IR spectra of the samples for discriminating the samples and extracting the specific bands of 15-AcDON/ZON.

For 15-AcDON with the concentration 0-100 ppm and 0-1ppm, MSC + mean center were used as preprocessing methods.

For ZON with the concentration 0.-10ppm, Baseline correction+ MSC + mean center were used as preprocessing methods.

For ZON with the concentration 0.1-2.25 ppm, Detrend (3^{rd} order) + first derivative (order:1, 21 points) + mean center were used as preprocessing methods.

3.3 Results and discussion

3.3.1 IR bands assignment of 15-AcDON and ZON

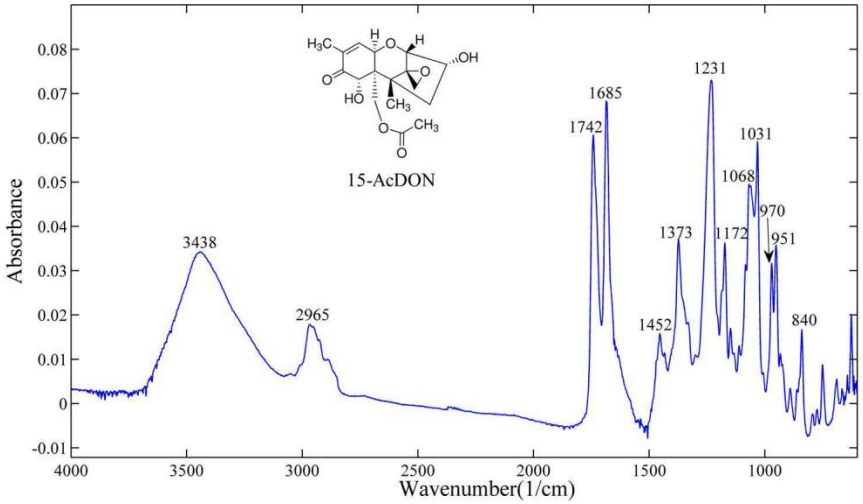

Figure 3.1 IR spectrum of 15-AcDON standard

Figure 3.1 shows IR spectrum of pure 15-AcDON ranged from 4000 to 600 cm^{-1}. Two broad bands were found at 3438 cm^{-1} and 2965 cm^{-1}, which results from the stretching vibrations of O-H and aliphatic C-H, respectively (Peiris et al., 2012). The adjacent peaks at 1742 cm^{-1} and 1685 cm^{-1} were due to ester C=O stretching and conjugated ketone stretching vibration, respectively (Mossoba et al., 1996). The bands with the peaks at 1231 cm^{-1} and 1031 cm^{-1} are due to asymmetric and symmetric stretch of ester (O) C-O, and the peak at 1068 cm^{-1} is due to the RCH-OH stretching (Young et al., 1994; Mossoba et al., 1996; Peiris et al., 2012). Epoxide ring is mainly the source of the toxicity of Trichothecenes. The IR absorptions located at 970 cm^{-1} and 951 cm^{-1} were assigned to epoxide ring stretch (Grove et al., 1988). Mossoba et al (1996) and Young et al (1994) analyzed the mid-infrared spectra of some trichothecene mycotoxins. However, they did not detect 15-AcDON. Grove et al. (1988) noticed three main infrared bands of 15-AcDON at 3450, 1745 and 1685 cm^{-1}, but they did not show the spectrum and give assignment for the bands. The assignment for C=O groups in this study well agreed with the results by Mossoba et al.(1996), Young et al.(1994) and Grove et al.(1988). These assignments are summarized in Table 3.1.

Table 3.1 IR band assignments of 15-AcDON

Wavelength(cm^{-1})	Functional group	Mode of vibration
3438	O-H	stretch
2966	Aliphatic C-H	stretch
1742	ester C=O	stretch
1685	conjugated ketone C=O	stretch
1453	Aliphatic C-H	bend
1373	CH3 in acetate	symmetric bend
1231	Ester(O)C-O	asymmetric stretch
1172	C-O-C in substituted 6-membered ring	asymmetric stretch
1068	RCH-OH	stretch
1031	ester (O)C-O	symmetric stretch
951,970	epoxide ring C-O	asymmetric stretch

Figure 3.2 displays the IR spectrum of ZON in the range of 1800-600 cm^{-1}. The IR bands of ZON was assigned according to (Cole, 2012; Pohland et al. 1982; Chakrabarti et al., 1986; Fotso, 2003) and ZON simulation spectrum by Winmostar software. One of the most important groups of absorptions is that of the carbonyl moiety. The IR absorption of lactone carbonyl group in the fourteen-membered ring of ZON was located at 1750-1704 cm^{-1} as a shoulder of the band of 1690 cm^{-1}. The bands at 1690 cm^{-1} and 1645 cm^{-1} were assigned to stretching vibrations of ketone and olefinic C=C groups, respectively, while the bands at 1608 cm^{-1} and 1587 cm^{-1} were resulted from the aromatic ring. The lactone (O)C-O presents peaks at 1310 and 1257 cm^{-1}. The bands in the 1217-800 were difficult to give assignment, because Olefinic absorption is usually accompanied by bands in the region 1000-800 cm^{-1}, and aromatic absorption by bands in the regions 1225-950 cm^{-1} and 900-800 cm^{-1}, and ester (O)C-O absorb in the range

1350-1000 cm^{-1}. In order to give accurate bands assignment, the simulation of the ZON IR bands was performed by using Winmoster software, which shows each IR bands resulted from the functional groups of ZON. It was found that the bands at 1215-1046 cm^{-1} were resulted from the stretching vibrations of the aromatic ring, while the bands at 1018 cm^{-1} and 970 cm^{-1} were caused by the stretching vibrations of C-O of the aromatic ring. The bands of 846 cm^{-1} and 706 cm^{-1} were related to the bending vibrations of the fourteen-membered ring. The above bands assignment was summarized in the Table 3.2.

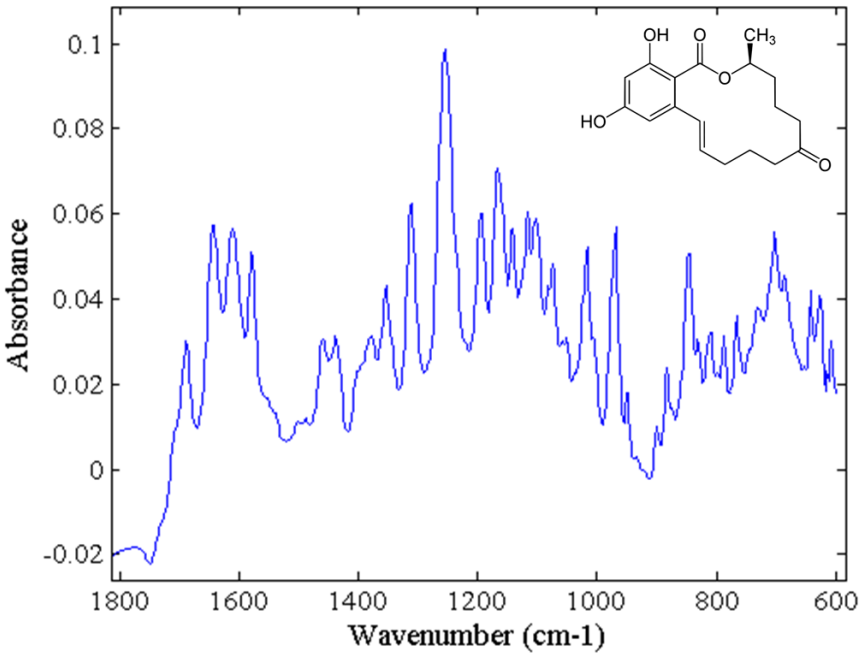

Figure 3.2 IR spectrum of ZON

Table 3.2 IR band assignments of ZON

Wavelength(cm^{-1})	Functional group	Mode of vibration
1750-1704, shoulder	(O)C=O	stretch
1690	Ketone C=O	stretch
1645	olefinic C=C group	stretch
1608, 1587	aromatic ring	stretch
1310, 1257	lactone (O)C-O	bend
1215-1046	aromatic ring	stretch
1018, 970	C-O of aromatic ring	stretch
846, 706	aromatic ring	bend

3.3.2 Characterization of ATR-FTIR spectra of corn oil contaminated with 15-AcDON

Raw spectra of the FFO samples in Figure 3.3 (a) show IR absorbance differences with concentrations of 15-AcDON in the 1080-980 cm^{-1} range, which were further resolved after MSC (Figure 3.3 (b)). MSC (multiplicative scatter correction) is a mathematical transformation method of the spectra used to remove slope variation and to correct for scatter effects. Figure 3.3 (b) presented that the samples were grouped into 100ppm, 10ppm, 1ppm and 0-0.1ppm according to the bands intensity in the 1080-980 cm^{-1}. According to the bands assignment for 15-AcDON, this wavenumber range corresponded to the stretching vibrations of ester (O)C-O and RCH-OH of 15-AcDON.

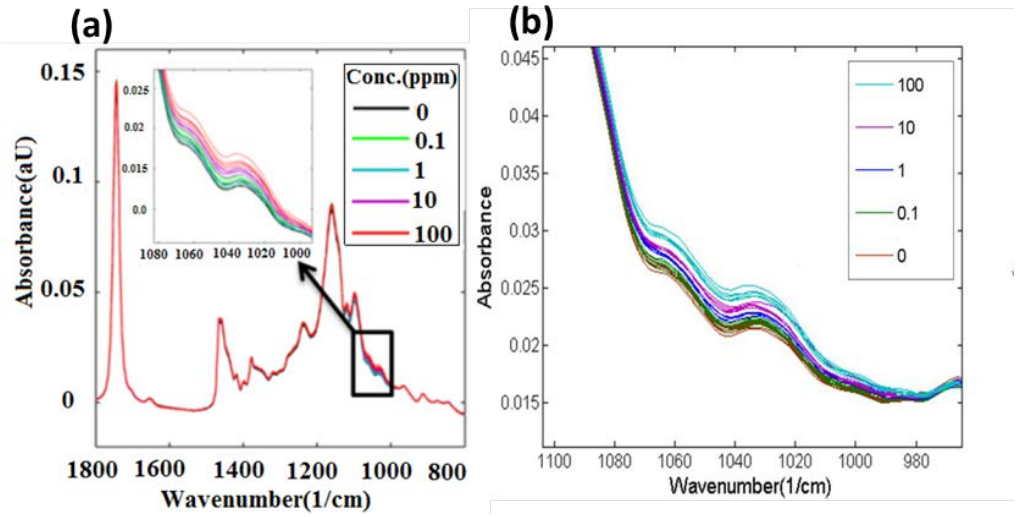

Figure 3.3 Raw (a) and MSC corrected (b) IR spectra of corn oil contaminated with 15-AcDON at different levels

PCA was performed in the 1150-900 cm^{-1} region to discriminate the samples with different concentrations of 15-AcDON. The spectra were preprocessed by MSC and mean centering. As shown in Figure 3.4(a), the first two principal components PC1 (87.83%) and PC2 (4.28%) explained around 92% of the total variation. The corn oil samples were grouped into 100ppm, 10ppm and 0-1ppm separately along PC1 scores, suggesting that PC1 score was related to 15-AcDON when the levels is higher than 10 ppm. Specifically, the 10ppm and 100ppm samples were located on the negative side with clear separation between each group, while the 0, 0.1 and 1ppm samples were located on the positive side with poor discrimination between each concentration level along PC1.

In the loading plot of PC1 (Figure 3.4(b)), two negative bands around 1003-1044 cm^{-1} and 1084-1102 cm^{-1} have strong influences on PC1 scores, which contributed to the discrimination between 10ppm and 100ppm samples. The 1003-1044 cm^{-1} band with a broad peak at 1025 cm^{-1} could be assigned to the band of 1031 cm^{-1} (symmetric stretching vibration of ester (O)C-O of standard 15-AcDON (Figure3.1), while the 1084-1102 cm^{-1} with a peak at 1095 cm^{-1} might be related to the change of the vibration C-C-O link derived from secondary alcohols(typically found at 1100 cm^{-1}) of corn oil due to the variation of 15-AcDON levels (Guillen et al., 1997). In addition, PCA was

performed to the 1003-1044 cm^{-1} and 1084-1102 cm^{-1} separately. The same discrimination result was found at 1003-1044 cm^{-1}, but not found at 1084-1102 cm^{-1}, suggesting that the specific bands of 15-AcDON with high concentration of 10-100ppm were at 1003-1044 cm^{-1}, which directly corresponded to the 15-AcDON levels.

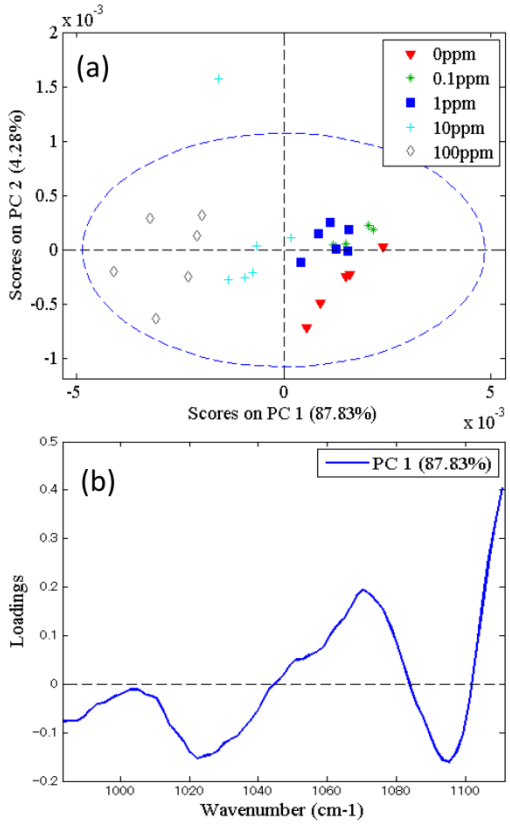

Figure 3.4 Scores and loading plots for corn oil contaminated with 15-AcDON at 0, 0.1, 1, 10 and 100ppm

The corn oil samples with lower concentration of 15-AcDON at 0, 0.1 and 1ppm were not discriminated in the above analysis, probably because of the following two reasons: (a). compared to the absorbance difference caused by the higher concentration of 10 and 100ppm, the absorbance of the lower concentration of 0, 0.1 and 1ppm was as low as noise. (b). the IR bands difference caused by the higher concentration of 10 and 100 ppm was different from that by the lower levels, because IR bands intensity with the higher concentration was directly corresponding to the concentration levels, while the lower one was caused by changes in the principal

constituents of corn oil from changes of 15-AcDON levels. In order to confirm this hypothesis, PCA was performed in the range of 1200-1060 cm^{-1} for corn oil with lower concentration of 15-AcDON at levels of 0, 0.1 and 1ppm for further discrimination.

Figure 3.5 shows that the first three principal components PC1(42.67%), PC2 (36.11%)and PC3(12.40%) represent 91% of the total variation. PC1 (Figure 3.5(a)) explained more variation compared to other two PCs, but the variation did not correspond to the concentration of 15-AcDON. PC1 loadings (Figure 3.5(b)) show a negative peak at 1100-1095 cm^{-1} which might be related to the vibration of C-C-O link derived from secondary alcohols(typically found at 1100 cm^{-1}) of corn oil (Guillen et al., 1997). In the PC2 and PC3 scores plot (Figure 3.5(c)), it was observed that the corn oil samples were classified into three groups according to the concentration of 15-AcDON, and the concentration was increased along the diagonal of the plot. Specifically, most of the 1ppm samples were located on the negative sides of PC2 and PC3, and the 0ppm samples were on the positive sides of PC2 and PC3.

In the loadings plot of PC2 and PC3, it was found that PC2 and PC3 both have negative loadings at 1090-1075 cm^{-1}, which was probably resulted from the vibration C-O link (typically found at 1150-1060 cm^{-1}) and/or rocking vibration of $(CH_2)_n$ (typically found at 1100-1000 cm^{-1}) of corn oil due to the variation of 15-AcDON levels (Guillén et al., 1998; Abramovic et al., 2003), while PC2 and PC3 both have positive loadings in the 1112-1095 cm^{-1}, which corresponded to the loading of PC1.

Hence, the stretch of C-O of corn oil was probably changed caused by the variation of levels of 15-AcDON. The specific bands of 15-AcDON with lower levels of 0-1ppm were at 1090-1075 cm^{-1}.

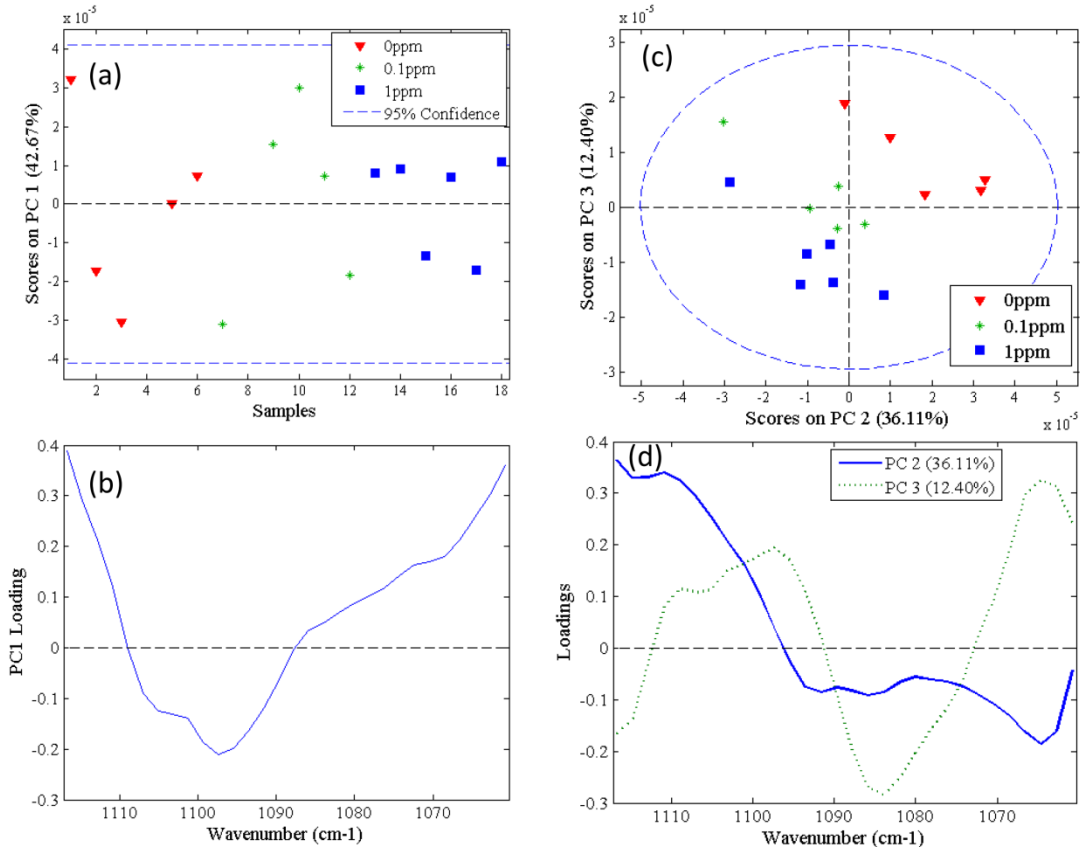

Figure 3.5 Scores and loading plots for corn oil contaminated with 15-AcDON at 0, 0.1, 1ppm

3.3.3 Characterization of ATR-FTIR spectra of corn oil contaminated with ZON

Because the intensity difference of IR spectra caused by ZON is not so visible because of the low concentration, spectral subtraction was performed. Figure 3.6 shows the subtractive spectra between ZON level of 0ppm and 0.1ppm, 0.5ppm, 1.2ppm, 2.25ppm, 10ppm in the corn oil samples. The absorbance between 10ppm and other samples was obviously different in the fingerprint range of 1500-1000 cm^{-1}. Thus, this region was selected for further analysis by PCA.

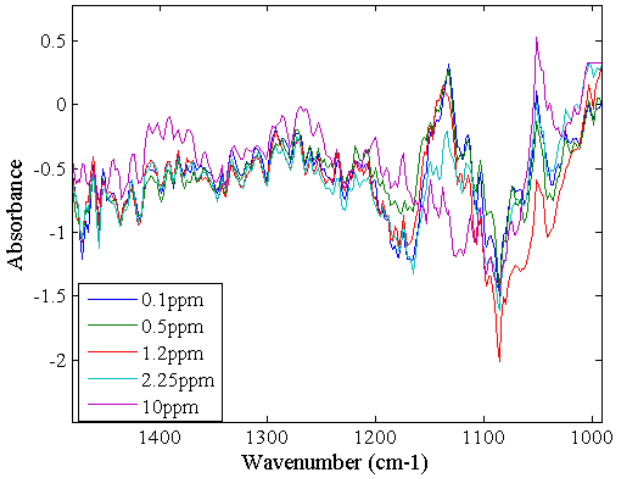

Figure 3.6 Averaged subtractive IR spectra of corn oil samples with ZON at 0.1 ppm, 0.5 ppm, 1.2ppm, 2.25ppm from the averaged spectrum of 0ppm sample

Baseline correction and MSC were used as preprocessing methods for PCA in the range of 1500-1000 cm^{-1}. The first two PCs explain more than 91% of the total variance, but only PC1 were considered for the successive analysis because it showed to be able to separate the samples. As shown in Figure 3.7(a), a distinct separation was observed between 10ppm and other levels along PC1 score. Specifically, the 10ppm samples were located on the negative side, while other samples were on the positive side. PC1 loading presents strong contribution in the range of 1230-1150 cm^{-1} with peak at around 1170 cm^{-1}, which corresponds to the stretching vibration of aromatic ring of ZON. The positive loading in the range of 1150-1050 cm^{-1} might be related to the vibration of C-C-O link derived from secondary alcohols (typically found at 1100 cm^{-1}) (Guillen et al., 1997), or the C-O bonds of fatty acids (typically found at 1097 cm^{-1}) of corn oil (Guillen et al., 1998), which resulted into the non-discrimination between 0.1 ppm, 0.5 ppm, 1.2 ppm and 2.25ppm. Therefore, the specific bands of ZON with higher concentration levels were located in the range of 1230-1150 cm^{-1}, caused by the stretching vibration of aromatic ring of ZON.

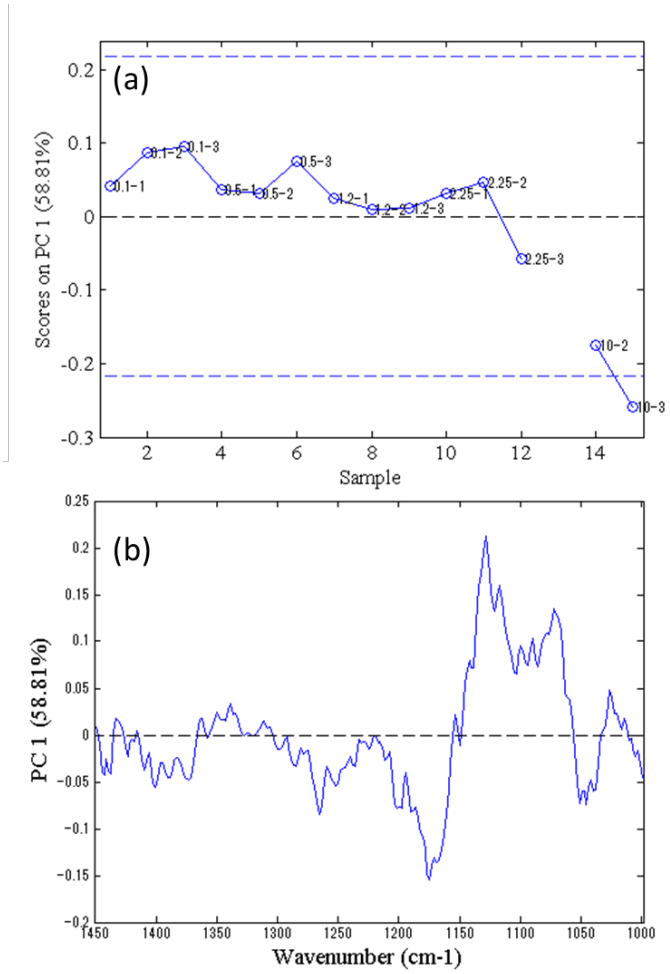

Figure 3.7 Scores and loading plots for corn oil contaminated with ZON at 0.1 ppm, 0.5 ppm, 1.2 ppm, 2.25ppm and 10ppm

In order to identify the specific bands of ZON with lower levels, detrend (third order) and first derivative (order:2, 21 points) were applied to the difference spectra of 0ppm from 0.1ppm, 0.5ppm, 1.2ppm, 2.25ppm(Figure 3.8). Figure 3.8 presents that the bands intensity was increased with the concentration levels of ZON in the range of 1800-1500 cm^{-1}. Hence, this region was selected for PCA analysis.

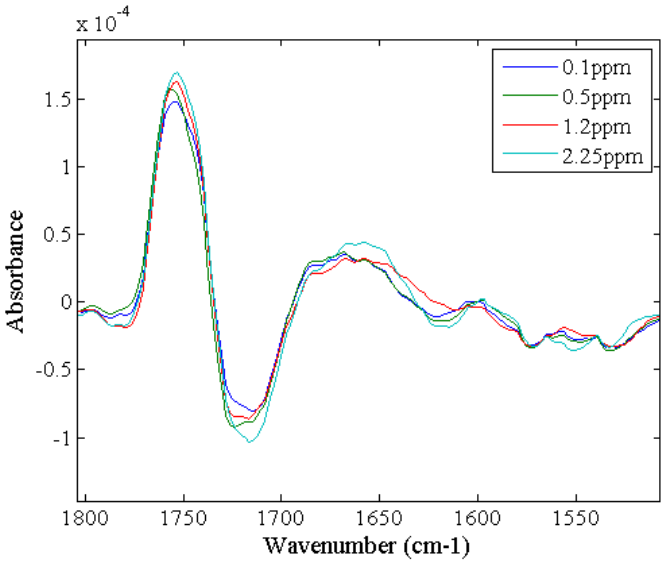

Figure 3.8 Averaged subtractive IR spectra of corn oil samples with ZON at 0.1, 0.5, 1.2 and 2.25ppm from the averaged spectrum of 0ppm sample

Figure 3.9(a) shows that the first two principal components PC1 and PC2 represented 97% of the total variation, and the 2.25ppm, 1.2ppm and 0.1ppm, 0.5ppm samples were separated into three groups. In addition, the concentration of ZON of the samples was increased along the diagonal of PC1 and PC2, and the 2.25ppm samples were classified on the positive sides of PC1 and PC2. From the loadings of PC1 and PC2, it can be seen that both PC1 and PC2 have positive values in the range of 1760-1730 cm^{-1}. Because of the low concentration of the samples, this range was possibly resulted from the ester C=O bonds of corn oil due to the addition of ZON rather than the lactone C=O of ZON. This hypothesis and band assignment was in agreement with previous investigations by Abramovic et al. (2007) and Kos et al. (2004) for DON determination by ATR-FTIR spectroscopy. Hence, the range at 1760-1730 cm^{-1} could be the specific band related to the C=O bonds of corn oil.

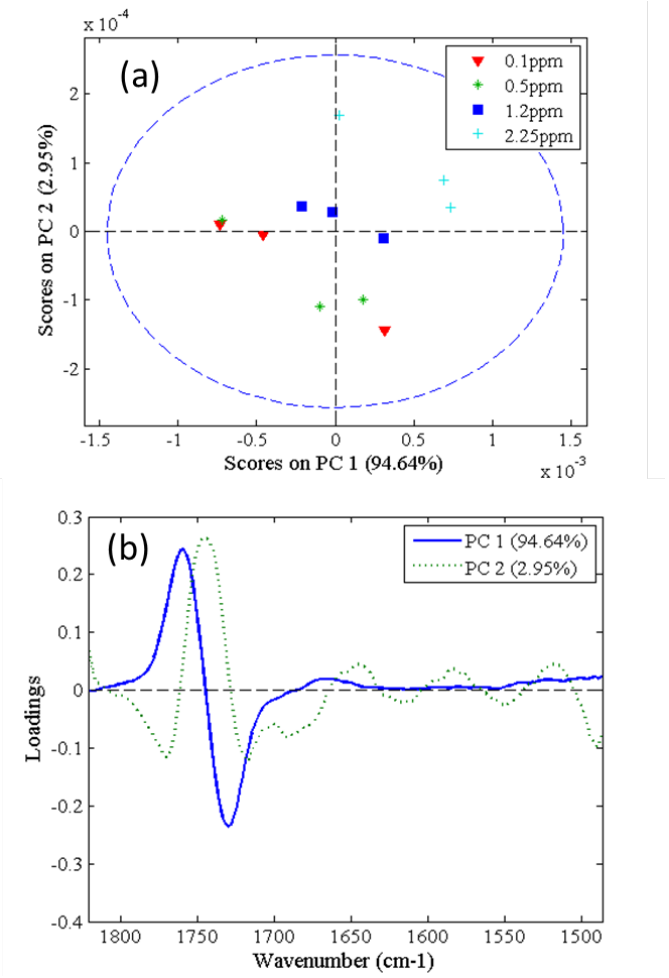

Figure 3.9 Scores and loading plots for corn oil contaminated with ZON at 0.1 ppm, 0.5 ppm, 1.2 ppm, 2.25ppm

3.3.4 Comparison with previous investigations

In this study, the specific bands of 15-AcDON and ZON were identified in different regions for the high and low concentration levels in corn oil. For 15-AcDON, the specific bands of the high concentration were identified at 1044-1003 cm^{-1}, and the specific bands of the low levels were found at 1090-1075 cm^{-1}. For ZON, the specific bands of the high concentration were revealed at 1230-1150 cm^{-1}, and the specific bands of the low levels were located at 1760-1730 cm^{-1}. In fact, previous studies have found the similar results to that of this study. De Girolamo et al.(2009) observed the specific NIR bands of 13ppm DON in wheat were located at around 6917 cm^{-1} and 5251

cm^{-1}, but the bands were not identified at lower levels for the PLS regression models.

For the high concentrations of the mycotoxins, the specific bands of 15-AcDON and ZON were assigned respectively to C-O of 15-AcDON and the aromatic ring of ZON. This band assignment was consistent to the findings from previous investigations on the interpretation of IR and NIR bands of DON. Peiris (2009) assigned the specific NIR bands of DON of 1300-1500 nm to the first overtone of C-H of DON, and of 1700-2000 nm to the second overtone of –C=O and R-OH. Peiris et al (2012) assigned the IR band of 1160 cm^{-1} of wheat kernels to 1167 and 1150 cm^{-1} of DON with high concentration.

For the low concentrations of the mycotoxins, the specific bands of 15-AcDON and ZON were attributed respectively to the C-O and ester C=O of corn oil. The previous studies were helpful for further interpreting the results of the low concentrations of 15-AcDON and ZON. It has been found that some mycotoxins interacted with the compositions of foods. Specifically, aflatoxin M1 in cheese has strong interaction with the protein of cheese (Mendonça and Venâncio, 2005). Fumonisin B1 has strong interactions with corn matrix during the alkaline cooking (Burns, 2008). On the other hand, the previous studies on high mycotoxin determination by IR or NIR spectroscopy did not assign the bands to the functional groups of the mycotoxin, because the studies used naturally contaminated materials, making the bands assignment difficult. However, this study artificially contaminated corn oil, making the bands assignment and interpretation possible. Therefore, the specific bands of 15-AcDON might be attributed to the interaction between 15-AcDON molecules and C-O of corn oil, while the specific bands of ZON could be assigned to the interaction between ester C=O of corn oil and ZON molecules.

3.4 Conclusions

The 15-AcDON or ZON contaminated corn oil were investigated using ATR-FTIR spectroscopy and PCA. The contaminated oils were discriminated according to the concentration of 15-AcDON or ZON. The specific bands of 15-AcDON or ZON with higher and lower concentration were identified in different regions. Higher levels corresponded to the functional groups of the mycotoxins, while lower concentrations were related to the interaction between the mycotoxins and composition of corn oil. Hence, before the determination of the mycotoxins, the specific bands of the higher and

lower concentrations should be firstly investigated for wavenumber selection. Besides, the suitable concentration range for modelling development could be selected according to the specific bands regions of mycotoxin concentration.

CHAPTER IV

DETERMINATION OF 15-ACETYLDEOXYNIVALENOL IN CORN OIL BY ATR-FTIR SPECTROSCOPY AND CHEMOMETRICS

4.1 Introduction

Mycotoxins are toxic metabolites of filamentous fungi, commonly present as contaminants in different foods, especially in cereals and cereal-based products. These contaminants can occur along the entire food chain, and thus adversely affect the health of both humans and domesticated animals.

Recently, considerable attention has been paid to the problem of mycotoxin contamination in edible oil especially the contamination with aflatoxins and trichothecenes (Bordin et al., 2014; Schollenberger et al., 2008). In the past, mycotoxin contamination in edible oil has not been recognized as a practical hazard because mycotoxins could be eliminated by an alkali treatment of chemical refining (Kamimura et al., 1986). However, mycotoxins have been detected in refined oil (Bordin et al., 2014; Schollenberger et al., 2008). It was found that the mycotoxins elimination by alkali treatment was less efficient for trichothecenes including 15-Acetyldeoxynivalenol (15-AcDON) compared to aflatoxins. After alkali treatment, aflatoxins B1 and B2 were reduced more than half of the initial contamination level in the first 2 min, but the trichothecenes (including 15-AcDON) were reduced by only 50 % in 8 min (Bordin et al., 2014). Furthermore, it has been reported that 15-AcDON has significantly higher contamination levels compared with other trichothecenes in refined oil (Schollenberger et al., 2008).

15-AcDON, together with 3-AcDON (3-acetyldeoxynivalenol), is acetylated derivative of Deoxynivalenol (DON), which belongs to trichothecene mycotoxin produced by *Fusarium spp.*. 15-AcDON raises attention on account of highly acute toxicity and cytotoxicity to humans and domestic animals, and great damage to agricultural grains, such as wheat and corn (Eriksen et al., 2004; Forsell et al., 1987). It has been reported that the contamination levels of 15-AcDON had high correlation with that of DON in cereals (Yoshizawa and Jin, 1995). And the contamination level was frequently ranged from 0.01ppm to 5ppm (Placinta et al., 1999; Perkowski et al., 1990). According to the frequent contamination and various toxicities of 15-AcDON and DON,

European Committee (EC) has set the maximum level of 1ppm for DON in food (Commission Regulation (EC) No 1881/2006). JECFA (2001, 2010) set the Provisional Maximum Tolerable Daily Intake (PMTDI) to the group of DON and its acetylated derivatives 3-Ac-DON and 15-Ac-DON at 1 μg/kg body weight (b.w.) per day.

Most of the analytical methods for detecting 15-AcDON in food involve two steps including extraction and quantification (Visconti et al., 2007; Lattanzio et al., 2009). Basically, the instrumental methods including gas chromatography (GC), high performance liquid chromatography (HPLC) and mass spectrometer (MS) are adopted for the precise detection, but they are time-consuming and require operation experience and expertise.

Mid infrared spectroscopy (MIR) has become a well-accepted technique and has received wide acceptance in food industry, mainly because it is low-cost, environment-friendly and generally requires very few operations and no sample extraction prior to analysis(Maggio et al., 2009). Recently, attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy has gained wide acceptance in different fields in consideration of its advantages over other techniques, such as the ability to measure the solid and liquid samples without any pretreatment. This characteristic makes ATR-FTIR spectroscopy especially attractive for straightforward, speedy characterization of the compositions or contaminants in food (Griffiths et al., 2007). ATR-FTIR spectroscopy is able to reveal information about the molecular bonds and hence provides details of molecular structure of samples. Moreover, chemometric methods, especially multivariate data analysis, can extract spectral features and establish a relationship between two data matrices. The first data matrix contains concentration information and the second one contains the spectral information of the analyzed samples (Kuligowski et al., 2008). The combination of ATR-FTIR and chemometric methods has been successfully applied for characterization and determination of contaminants of food, such as pesticides and mycotoxin (Quintás, et al., 2003; Singh et al., 2011). So far, ATR-FTIR spectroscopy has been applied in grains, dried vine fruits, nuts for determining mycotoxins, such as aflatoxins, deoxynivalenol, and ochratoxin A. Moreover, multivariate methods, such as partial least squares (PLS) and multiple linear regression (MLR), have been used in determining mycotoxins of foods by using FTIR spectroscopy with the spectral region from 1800 to

800 cm^{-1} or 4000-600 cm^{-1}. However, in the previous investigations, the selected spectral regions were so wide and maybe include some useless information, which maybe decreased the performance of the models.

The aim of this study was to evaluate the feasibility of ATR-FTIR spectroscopy for detection of the 15-AcDON mycotoxin in corn oil. In order to find the optimal model for accurate and precise quantification of 15-AcDON, multivariate calibration methods, including partial least squares (PLS), multiple linear regression (MLR) and principal component regression (PCR), were firstly performed with different preprocessing methods in the wavenumber region of 4000-600 cm^{-1}. Different variable selection methods were used to develop the optimal models. For PLS, the optimized FTIR spectral regions of 15-AcDON in corn oil were revealed by using genetic algorithm (GA), and interval partial least squares (iPLS). For MLR, the specific FTIR bands of 15-AcDON were selected by using GA and stepwise variable selection methods. And for PCR, interval principal component regression (iPCR) was used for choosing the optimal model. For each kind of model, the performance of the chemometric models established by using the optimized spectral regions was compared to that of models based on the whole region (4000-600 cm^{-1}).

4.2 Materials and methods

4.2.1 Sample preparation

15-AcDON standard and corn oil were purchased from Wako Pure Chemical Industries, Ltd. Japan.

Standard solution: 15-AcDON in pure crystalline form was dissolved in pure methanol to prepare 100ppm 15-AcDON-methanol solution.

Artificial contamination: 100ppm artificially 15-AcDON-contaminated corn oil was prepared by adding the resulting 15-AcDON-methanol solution into corn oil, followed by removing the residual methanol of corn oil in a desiccator with reduced pressure.

A total of 57 corn oil samples with 15-AcDON concentrations between 0.1 and 2.25 ppm were prepared by diluting the artificially contaminated corn oil with pure corn oil. The concentration of 15-AcDON was prepared at 0.1-2.25 ppm in corn oil, according to Chapter III and maximum allowed level by EC. In addition, 0.1-2.25ppm could cover the contamination level in previous studies. The total 57 samples were automatically split

into 36 samples for calibration development and 21 samples for model validation by using “Onion” method with replicates together. The “onion” method keeps outside covariance samples plus random inner-space samples.

4.2.2 FTIR spectroscopy measurement

IR Spectra were collected using MB3000 FT-IR spectrometer (ABB Inc. Canada) with a MIRacle ZnSe45° attenuated total reflectance (ATR) accessory (PIKE Tech. USA) in the 4000-600 cm^{-1} region. The absorbance of samples was measured at a resolution of 4 cm^{-1} and with 32 scans. Air-conditioner in the laboratory turned on to keep a stable room temperature of 25℃ and a steady level of humidity.

For 15-AcDON contaminated corn oil, the absorbance spectrum was collected against a background obtained with a dry and empty ATR cell. Before recording each spectrum, the ATR crystal was cleaned with a KimWipe tissue (Kimberly-Clark Corporation, USA) and 70% ethanol solution to minimize sampling error, the mean of the three spectra were collected from the same corn oil sample for the following analysis step.

4.2.3 Spectral data preprocessing

Several preprocessing methods, such as MSC, detrend (order: 0-5), SNV, Normalize, first and second derivative (order: 1-3, window width: 3-51), mean center, and their combinations, were compared for spectral data before developing models.

4.2.4 Development of 15-AcDON quantification models

The multivariate models multiple linear regression (MLR), principal components regression (PCR), and partial least squares regression (PLSR) were developed in this study for quantitative determination of 15-AcDON in corn oil. In the model development, actual 15-AcDON concentration values were correlated with the FTIR spectra. The PCR and PLSR are spectral decomposition techniques, which find the most relevant factors to explain the variance in the data set. The PCR concentrates on the variance in the spectral data, while the PLSR regression technique compares the covariance between the spectral data and 15-AcDON concentration (Lee, et al., 2014a). Multiple linear regression approach (MLR) is the simplest calibration method by connecting the

concentration of 15-AcDON to the peak's height.

The MLR, PCR, PLSR models were cross-validated using venetian blinds method. The models were evaluation according to the correlation coefficient of determination of cross validation (R^2_{CV}). The final multivariate models were validated externally according to the root mean standard error of prediction (RMSEP) and correlation coefficient of determination (R^2_P) with prediction dataset (Lee, et al., 2014a).

4.2.5 Selection of spectral regions

Five methods were used for spectral selection: GA-PLS, iPLS, GA-MLR, stepwise-MLR and iPCR. The selected spectral regions were determined by referring to the lowest root mean square errors of cross-validation (RMSECV).

1. Genetic algorithm (GA)-PLS and GA-MLR

The variables selection for multivariate calibration can be considered as an optimization problem. The genetic algorithm (GA) is a heuristic optimization technique that employs a probabilistic process inspired by Darwin's theory of natural selection. In this study, for GA, random subsets of the whole spectral region are chosen according to the lowest RMSECVs in partial least squares (PLS) regression or MLR model. Among the entire processes of GA, firstly, random subsets of the whole spectral region are generated; secondly, the RMSECV of each individual subset is evaluated, and the half of the subsets with higher RMSECV is discarded; thirdly, to replace the discarded subsets, in this study, the retained subsets are bred by double cross-over breeding, and the mutation is allowed; finally, the second and third steps are repeated until ending criteria are met. In the process of GA-PLS or GA-MLR algorithms implementation, the control variables of the GA were set to: initial population size 128, crossover rate 0.5, and probability of mutation 0.01; the stopping criterion for the GA was 100 iterations.

2. iPLS and iPCR

The interval PLS (iPLS) and interval PCR (iPCR) algorithms applied here were described by Nørgaard, et al. (2000). The basic principles of the algorithms are as follows. First, the full-spectrum is divided into equidistant subintervals (variables-wise). Next, PLS models or PCR models for all possible combinations of two, three, or four subintervals are constructed, and the RMSECV is calculated for each model of different combination of subintervals. The combination of subintervals is selected according to

the lowest RMSECV.

3. Stepwise MLR(MLR-step)

In the algorithm for stepwise multiple linear regression (MLR-step) (Forina et al., 2007), original variables are selected iteratively according to the RMSECV of the cross-validation.

The GA-PLS, GA-MLR, iPLS, iPCR and MLR-step were carried out by PLS_Toolbox (Eigenvector Research Inc., WA, USA) in MATLAB software (The MathWorks Japan Inc., Tokyo, Japan)

4.2.6 Statistical analysis

The abnormal spectra were identified using residual statistic (Q statistic), establishing a confidence limit value of 95%, such that the samples whose Q residual were greater than the set value.

The performance of the developed models were evaluated by RMSEC (root mean square error of the calibration), RMSECV (root mean square error of the cross-validation), RMSEP(root mean square error of the prediction), R^2 (coefficient of determination). And their formulas are as follows:

$$RMSEC=\sqrt{\frac{\sum_{i=1}^{N}(Y_i^{act}-Y_i^{pre1})^2}{N}} \quad (4.1)$$

$$RMSECV=\sqrt{\frac{\sum_{i=1}^{N}(Y_i^{act}-Y_i^{pre2})^2}{N}} \quad (4.2)$$

$$RMSEP=\sqrt{\frac{\sum_{i=1}^{N}(Y_i^{act}-Y_i^{pre3})^2}{N}} \quad (4.3)$$

$$R^2=\frac{\sum_{i=1}^{N}(Y_i^{pre}-\overline{Y_i})}{\sum_{i=1}^{N}(Y_i^{act}-\overline{Y_i})} \quad (4.4)$$

Where, N is the number of samples, Y^{act} is the actual concentration level of 15-AcDON of corn oil samples prepared. Y^{pre1} is the predicted value of calibration models, Y^{pre2} is the predicted value of cross-validation models Y^{pre3} is the predicted value of validation models and $\overline{Y_i}$ is the average value. An R^2 with a value closer to 1 indicates a better fit of the model.

4.3 Results and discussions

4.3.1 ATR-FTIR spectral feature of 15-AcDON-contaminated corn oil

Figure 4.1 presents the calibration spectra after standard derivation (SD). It can be obviously seen that the samples have significant IR band variations at around 2900 cm^{-1}, 1740 cm^{-1} and the fingerprint region from 1600 to 800 cm^{-1}, which corresponded to the stretching vibrations of O-H, ester C=O, and the C=H, C-O bonds of corn oil. It was noticed that 610 cm^{-1} has a high SD value, which may be the noise caused by the FTIR spectrometer. Besides, the region of 4000-3800 cm-1 and 2700-1900 cm^{-1} has only noise except the region of 2400-2200 cm^{-1} caused by carbon dioxide (CO_2).

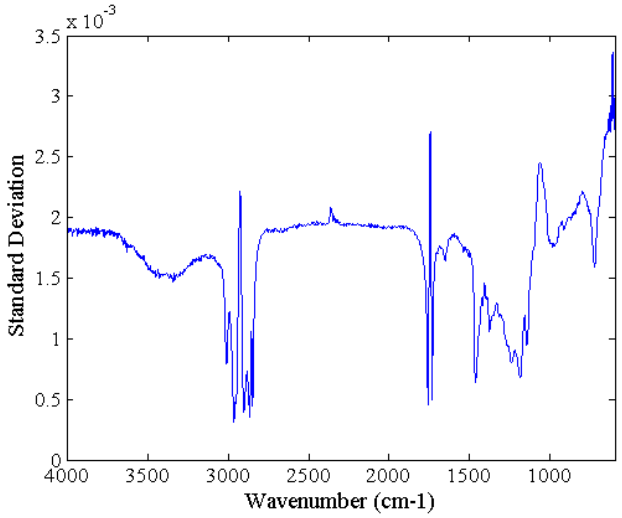

Figure 4.1 Standard deviation spectrum of 15-AcDON contaminated corn oil

4.3.2 Comparison of preprocessing methods

For comparing the preprocessing methods, PLS was performed with full spectral region. The following preprocessing methods were conducted: filtering methods (baseline correction (order: 1-3), detrend (order: 0-5), first derivative (order: 1-6, points: 3-55), second derivative (order: 2-6, points: 3-55), smoothing (points: 3-55)) and normalize methods (MSC(mean), MSC(median), SNV, normalize). Comparing the preprocessing methods was based on the following steps. Firstly, each single preprocessing method followed by mean centering (SMC) was performed. Secondly, after comparing the SMC with mean centering, the “superior” preprocessing methods were retained and combined for further comparison. The “superior” referred to the resulted models with relevant preprocessing methods have higher R_{CV}^2, fewer LV numbers, and lower RMSECV and RMSEP.

Table 4.1 presents the comparison results from SMC, MC, and combination of the superior methods. For PLS model, it was found that the first and second derivatives have lower R^2_{CV} and higher RMSECV and RMSEP values, but baseline correction and detrend significantly improved the model performance. Besides, smoothing the spectra also optimized the model. The poor performances of the first and second derivatives might be caused by the spectral noise generated by the algorithms. Therefore, first or second derivative combined with smoothing was performed. However, the results were still unsatisfactory. For the normalization methods, MSC (mean), MSC(median) and SNV generated comparable results, while Normalize had more superior model performance. All the superior methods were combined to optimize further the model. However, the combined methods did not promote the model performance. Finally, the detrend (0) followed by mean centering was used for the PLS model development.

Table 4.1 Comparison of preprocessing methods for PLS, PCR and MLR models

Model	Preprocessing methods	LV/PC number	R^2_{CV}	RMSECV	RMSEP
PLS	MC	8	0.548	0.471	0.440
	1D-MC	4	0.438	0.474	0.499
	2D-MC	6	0.459	0.482	0.493
	SG(0,3)-MC	7	0.559	0.465	0.425
	MSC(mean)-MC	7	0.587	0.451	0.486
	MSC(median)-MC	8	0.582	0.454	0.462
	SNV-MC	7	0.590	0.450	0.487
	Detrend(0)-MC	**7**	**0.670**	**0.360**	**0.400**
	BC(1)-MC	7	0.659	0.365	0.403
	Norm-MC	8	0.657	0.366	0.433
	SNV-SG(0,3)-MC	7	0.607	0.439	0.436
	MSC-SG(0,3)-MC	7	0.604	0.441	0.435
	BC(1)-SNV-MC	7	0.594	0.447	0.402
	Detrend(0)-MSC(mean)-MC	7	0.587	0.451	0.486
	Detrend(0)-MSC(median)-MC	7	0.549	0.470	0.490
MLR	BC-MC		0.623	0.380	0.793
PCR	SNV-MC	10	0.526	0.435	0.238

MC: mean center; 1D (a,b): first derivative(order, point); 2D (a,b): second derivative(order, point); SG(a,b): smoothing(order, point); BC: baseline correction; SNV: standard Normal variate ; Norm:normalize; MSC: Multiple Scatter Correction;

As a comparison, the same selection procedures were carried out for MLR and PCR. And it was found that baseline correction and SNV were superior for MLR and PCR respectively, compared to other preprocessing methods and their combinations.

4.3.3 Selection of spectral regions

Because the calculations of the whole spectral regions were difficult to be completed by a general personal computer, the spectra were initially selected based on the characteristics of the 15-AcDON standard and contaminated corn oil spectra. The beginning 800-600 cm^{-1} spectral region was deleted because the FTIR spectrometer generated more noise, which interfered the analysis. The regions of 2700-1900 cm^{-1} and 4000-3800 cm^{-1} were also omitted for reducing the noise, because both 15-AcDON and corn oil did not generate characteristic IR bands in these regions. Besides, the band of asymmetric stretching vibration of carbon dioxide (CO_2) at 2400-2200 cm^{-1} significantly deteriorated the models. Thus, the regions of 800-1900 cm^{-1} and 2600-3700 cm^{-1} were used separately for conducting the spectral selection methods (GA-PLS, GA-MLR, iPLS, iPCR and MLR-step). Besides, the combination of the separately specific regions from 800-1900 cm^{-1} plus 2600-3700 cm^{-1} was also conducted for comparison with the separately specific regions. The GA-PLS, GA-MLR, iPLS, iPCR and MLR-step with the window size of 10 spectral points, maximum LV of 10 were carried out.

Figure 4.2 presents the results for 1900-800 cm^{-1} spectral region, obtained by GA-MLR and GA-PLS, for 15-AcDON in corn oil. The included variables were selected based on the lowest RMSECV. It was apparent that when 130 and 162 variables (black square) were selected respectively by GA-PLS and GA-MLR, the RMSECVs reached the lowest values of 0.302ppm and 0.312ppm. Because variables which are used more are usually more useful in the regression and variables which show up less are less useful, in this study, the frequencies of the intervals lower than 0.2 were removed from the models. From Figure 4.2 (b) (d), the specific spectral regions could be easily found by using GA, which are shown in Table 4.2 (row 2 and 5).

For iPLS, iPCR, and MLR step, the interval numbers between 1 and 20 were firstly selected with the lowest RMSECV values. From Figure 4.3(a), 14, 13 and 11 intervals were chosen for calculation of iPLS, iPCR and MLR step, respectively. From Figure

4.3(b), (c), (d), RMSECV variations could be easily observed, and the intervals with lowest RMSECV values were selected for calculations of iPLS, iPCR and MLR step.

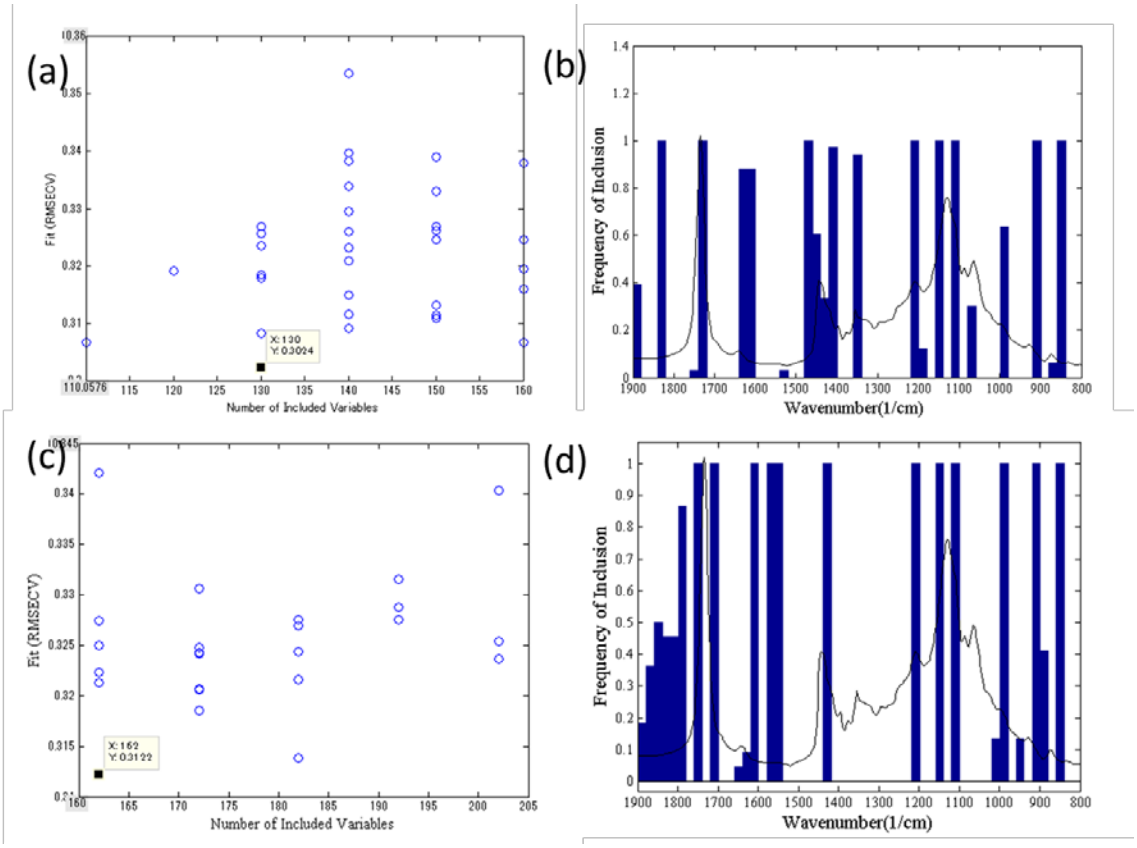

Figure 4.2 Diagnostic plots of 1900-800 cm^{-1} spectral region for 15-AcDON in corn oil by GA-PLS(a,b) and GA-MLR (c,d)

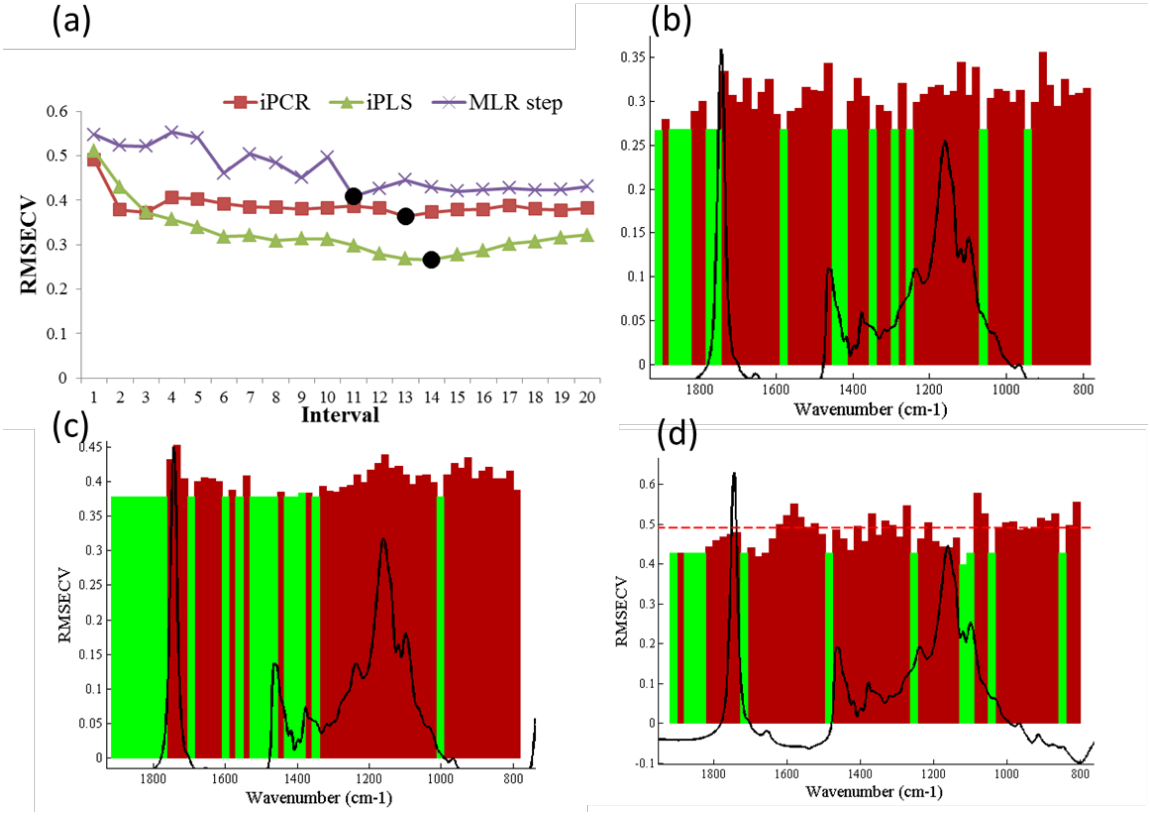

Figure 4.3 Diagnostic plots of 1900-800 cm^{-1} spectral region of corn oil for 15-AcDON by iPLS, iPCR and MLR step. (a) is the intervals selection results for iPLS, iPCR and MLR step. (b), (c), (d) are the selected intervals of iPLS, iPCR and MLR step, respectively.

Table 4.2 Cross-validation results of models for 15-AcDON in corn oil by specific spectral regions from 1900-800 cm^{-1} selected by GA-PLS, GA-MLR, iPLS, iPCR and MLR step.

Method	Spectral region	LV/PC numbers	R^2_{CV}	RMSECV
PLS	all	7	0.670	0.360
GA-PLS	1901-1884, 1843-1826, 1747-1730, 1496-1460, 1438-1421, 1380-1363, 1245-1228, 1188-1170, 1149-1132, 1033-1016, 956-939, 898-881	10	0.822	0.279
iPLS	1901-1888, 1878-1822, 1782-1745, 1589-1571, 1454-1417, 1357-1340, 1299-1282, 1261-1243, 1068-1051, 952-935	10	0.829	0.260
MLR	all		0.623	0.380
GA-MLR	1863-1845, 1805-1787, 1766-1749, 1728-1710, 1631-1614, 1593-1556, 1458-1440, 1245-1228, 1188-1170, 1149-1132, 1033-1016, 995-977, 956-919, 898-881		0.696	0.348
MLR step	1820-1803, 1762-1745, 1627-1610, 1569-1552, 1531-1514, 1473-1456, 1357-1340, 1261-1243, 1203-1186, 817 -798		0.577	0.409
PCR	all	10	0.526	0.435
iPCR	1901-1764, 1704-1687, 1608-1591, 1569-1552, 1531-1456, 1434-1379, 1357-1340, 1010-993	10	0.639	0.364

Table 4.3 Cross-validation and Prediction results of the models for 15-AcDON in corn oil by specific spectral regions selected by GA-PLS, GA-MLR, iPLS, iPCR and MLR step.

Method	Spectral region (cm^{-1})	LV(PC) numbers	R^2_{CV}	RMSECV	R^2_P	RMSEP
PLS	All	7	0.670	0.360	0.641	0.400
iPLS	1878-1822, 1782-1745, 1589-1571, 1454-1417, 1357-1340, 1299-1282, 1261-1243, 1068-1051, 952-935	10	0.829	0.260	0.748	0.436
GA-PLS	3663-3645, 3528-3510, 3412-3394, 3258-3240, 3199-3182, 3161-3143 3026-3008, 2929-2912, 2871-2854, 2833-2815, 2621-2603, 1901-1884, 1843-1826, 1747-1730, 1496-1460, 1438-1421, 1380-1363, 1245-1228, 1188-1170, 1149-1132, 1033-1016, 956-939, 898-881	9	0.789	0.291	0.847	0.211
MLR	all		0.623	0.380	0.532	0.793
MLR step	3652-3596, 3517-3500, 3421-3404, 2842-2825, 2804-2786, 2765-2748, 2669-2651, 1820-1803, 1762-1745, 1627-1610, 1569-1552, 1531-1514, 1473-1456, 1357-1340, 1261-1243, 1203-1186, 817 -798		0.728	0.330	0.608	1.097
GA-MLR	1863-1845, 1805-1787, 1766-1749, 1728-1710, 1631-1614, 1593-1556, 1458-1440, 1245-1228, 1188-1170, 1149-1132, 1033-1016, 995-977, 956-919, 898-881		0.697	0.347	0.624	0.476
PCR	all	10	0.526	0.435	0.569	0.438
iPCR	3710-3674, 3421-3384, 3305-3288, 3267-3249, 3035-3018, 2900-2864, 2842-2806, 2765-2729, 2688-2671, 2650-2594, 1917-1764, 1704-1687, 1608-1591, 1569-1552, 1531-1456, 1434-1379, 1357-1340, 1010-993	10	0.680	0.356	0.735	0.387

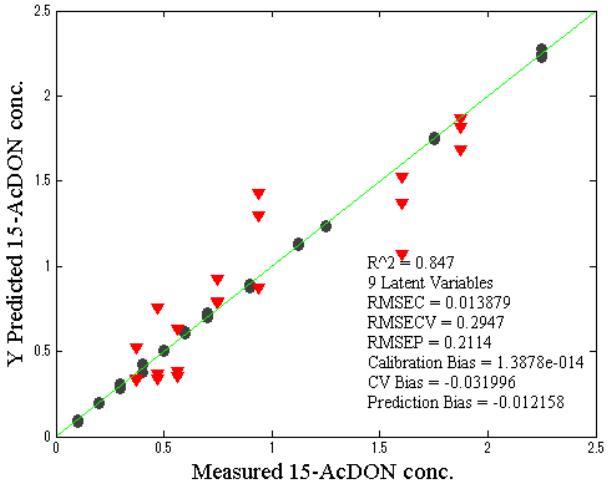

Figure 4.4 GA-PLS regression for prediction of 15-AcDON in corn oil

In this study, all the specific spectral regions in 1900-800 cm^{-1} and 3700-2600 cm^{-1} were determined by GA-PLS, GA-MLR, iPLS, iPCR and MLR step, respectively. And the models by the use of the combinations of the specific regions selected by the five methods were also developed, and the statistical results are shown in Table 4.3.

Compared with the full spectral regions, the specific regions selected by the five methods significantly optimized the models. For the cross-validation and prediction of 15-AcDON in corn oil, GA-PLS and iPLS provided better results using the optimized spectral regions combination (Table 4.3). iPLS had the coefficient of determination (R^2_{CV}) of 0.829, RMSECV of 0.260 with the latent variables number of 10 (see the rows 2-4), as well as RMSEP of 0.436, the coefficient of determination (R^2_P) of 0.748. Compared with iPLS, GA-PLS had the lower R^2_{CV}(0.789), higher RMSECV (0.291), but higher R^2_P of (0.841) and lower RMSEP (0.211). The results suggest that GA-PLS had better predictability. In addition, according to the spectral region selected by GA-PLS, the O-H, C=O, C-O of corn oil contributed to the performance of the PLS model.

In recent decades, mycotoxin detection by IR spectroscopy focused predominantly on the solid samples, such as grains (Kos et al., 2004; Abramovic et al., 2007). However, with the increasing concern on the hazard of mycotoxin in edible oil, it is urgent to develop a rapid and easy method for determining the mycotoxin in edible oil. This study investigated the possibility of detection of the 15-AcDON mycotoxin with the concentration from 0.1 to 2.25ppm in corn oil. Until now, aflatoxins, DON and

ochratoxins in grains, groundnut or fruit have been detected by IR spectroscopy (Kos et al., 2004; Abramovic et al., 2007; Galvis-Sánchez et al., 2007; Mirghani, 2001). Overall, the results with R_P^2 of 0.841 and RMSEP of 0.211ppm were better than that of Kos et al.(2004) and Abramovic et al. (2007). Kos et al.(2004) detected DON in corn by ATR-FTIR and PLS model with the level of DON at 0.3ppm-2.6 ppm. The best model obtained from their study was with correlation coefficient of cross validation 0.8111 and the RMSECV of 0.494 ppm. However, they did not provide the values of R_P^2 and RMSEP, due to lack of the prediction set. Abramovic et al (2007) obtained good MLR model for detection of DON in wheat by using ATR-FTIR, with the slope of 0.8693 and RMSEP of 1.523ppm, but the concentration was ranged from 2.51 to 12.14 ppm, which was higher than the maximum allowed level set by EU.

4.4 Conclusions

The PLS, MLR and PCR models for detection of 15-AcDON in corn oil were optimized by using preprocessing and spectral region selection methods. The PLS, MLR and PCR models with full spectra were firstly optimized by different preprocessing methods. The spectral regions for 15-AcDON in corn oil were selected by GA-PLS, GA-MLR, iPLS, iPCR and MLR step using the best preprocessing methods obtained. The models with specific bands selected by GA-PLS, GA-MLR, iPLS, iPCR and MLR step yield better predictability, compared to those obtained by the full region. In addition, the model developed using the specific regions by GA-PLS showed superior performance, because the statistical results demonstrated the lower RMSECV, RMSEP and higher $R_{CV,}^2$ R_P^2.

The results suggested that ATR-FTIR combined by GA-PLS was feasible for detection of 15-AcDON in corn oil.

CHAPTER V
DETECTION OF ZEARALENONE IN CORN OIL BY ATR-FTIR SPECTROSCOPY

5.1 Introduction

Zearalenone (ZON) is a non-steroidal oestrogenic mycotoxin produced by *Fusarium graminearum* and other *Fusarium* species, which are plant pathogenic fungi that infect a wide variety of cereals (Zinedine et al., 2007).

Recently, great attention has been raised on the ZON contamination in edible oil. Most of the mycotoxin could be eliminated or decreased during the oil production process, such as wet-milling, high temperature, alkali and acidity treatment (Li et al., 2014). Compared to aflatoxins and trichothecenes, ZON was robust to the refining process, and therefore became the most prevalent in refined oil, because ZON could be dissolved in oil directly, and the refining process decreased ZON to a lesser level (Li et al., 2014; Schollenberger et al. 2008; Kamimura et al., 1986). It has been reported that the contamination of ZON in refined oil was ranged from 5 to 440 ppb (Schollenberger et al., 2008; Li et al., 2014; EFSA, 2011). Because of the serious toxicity and high pollution rate of ZON, the European Commission, in 2007, has set the maximum allowed level of 400 ppb for refined corn oil (Commission Regulation (EC) No 1881/2006 of 19).

Several methods, such as TLC, HPLC, GC, HPLC-MS-MS, have been applied for detection of ZON in edible oil (Hagan and Tietjen 1975; Schollenberger et al., 2008; Siegel et al., 2010; Peng et al., 2009). The most frequently used techniques today are GC and HPLC, which are precise and suitable for laboratory analysis. However, the application of these methods is time-consuming, and requires a high level of experience and expertise, because the extraction and clean-up steps must be performed before separation and detection either by gas or liquid chromatography, which result in a low sample throughput (Kos et al., 2004).

Spectroscopic techniques are based on the identification of functional groups and the characterization of conformationally distinct structures in molecules. In recent years, mid and near infrared spectroscopy has found widespread use in the analysis of mycotoxin in cereals, spices and fruits (Singh & Jayas, 2011). FTIR has been

successfully used for detection of mycotoxin, such as deoxynivalenol, aflatoxins, and ochratoxins (Kos et al., 2004; Abramovic et al., 2007; Galvis-Sánchez et al., 2007).

Because of the high concern on the ZON of corn oil, and the potentialities of FTIR spectroscopy, the objectives of this study are to investigate the feasibility of FTIR for determining ZON in corn oil.

5.2 Materials and methods

5.2.1 Sample preparation

ZON in pure crystalline form was dissolved in pure methanol to prepare 100ppm ZON-methanol solution.

ZON contaminated corn oil was prepared by spiking the resulting ZON-methanol solution into corn oil, followed by removing the residual methanol of corn oil in a desiccator with reduced pressure.

A total of 66 samples with ZON concentrations from 0.0498ppm to 1.9 ppm were prepared. This concentration range can cover the majority of ZON concentrations found in commercial edible oil and legislation limit of most countries and organizations, and thus is considered suitable for developing the more useful and representative model. The total 66 samples were automatically split into 45 samples for calibration development and 21 samples for model validation by using “Onion” method with keeping replicates together. The Onion method firstly selects a ring of highly-unique samples based on distance, then leaves out a ring of the next-unique samples, then finally selects a random subset of samples inside the boundaries selected in the “onion”. This method could keep outside covariance samples plus random inner-space samples.

5.2.2 FTIR measurement

IR Spectra were collected using MB3000 FT-IR spectrometer (ABB Inc. Canada) with a MIRacle ZnSe45° attenuated total reflectance (ATR) accessory (PIKE Tech. USA) in the 4000-600 cm^{-1} region. The absorbance of samples was measured at a resolution of 4 cm^{-1} and with 32 scans. Air-conditioner in the laboratory turned on to keep a stable room temperature of 25℃ and a steady level of humidity.

For ZON contaminated corn oil, the absorbance spectrum was collected against a background obtained with a dry and empty ATR cell. Before recording each spectrum,

the ATR crystal was cleaned with a KimWipe tissue (Kimberly-Clark Corporation, USA) 70% ethanol solution to minimize sampling error, the mean of the three spectra were collected from the same corn oil sample for the following analysis step.

5.2.3 Data analysis

Data analysis was performed with PLS_toolbox 6.7.1 (Eigenvector Technologies, Wenatchee, WA, USA) in Matlab R2007b (Mathworks, USA).

For each sample, 3 repeat ATR measurements were averaged into one spectrum for the following analysis. PCA was used for outlier detection, classification and selecting the spectral regions for quantitative analysis PLS and PCR. For the quantitative analysis (PLS, PCR), different spectral regions and preprocessing methods were used for optimizing the models. The evaluation was conducted by using the correlation coefficients R^2_{CV} and RMSECV of cross-validation, and the correlation coefficients R^2_P and RMSEP of prediction.

5.3 Results and discussion

5.3.1 Spectral feature of ZON-contaminated corn oil

The preprocessing method used for IR spectra is first Derivative (order1, points: 29)-mean center, which enhanced characteristic IR absorption bands, improving predictive accuracy of the models. The mathematical preprocess is usually employed to remove scattering effects, to eliminate irrelevant chemical information and to extract only meaningful information from collected spectra. Figure 5.1 shows the difference between samples with different concentration levels of ZON. From this figure, it was noticed that the samples of 0.0498-3 and 1.9-2 were distinctly different with others, and might be outliers. Besides that, for other samples, some spectral similarities were observed among all the samples, indicating that some common major functional chemical groups coexisted in the corn oil samples. However, due to the variation of ZON concentration in corn oil, distinctive spectral differences in relative intensity and position of bands were found in several regions. Among ZON-contaminated corn oil samples, large differences were observed in the IR spectral ranges of 3071-2766 cm^{-1}, 1830-1661 cm^{-1}, 1577-980 cm^{-1} and 950-610 cm^{-1}, due to ZON effects on corn oil. The major IR bands of interest could be tentatively assigned based on previous literature

(Alexa et al., 2009; Guillén and Cabo, 1998; Coates, 2000). IR bands at 2995 cm^{-1}, 2876 cm^{-1} and 2773 cm^{-1} correspond to the C-H and O-H stretching modes, respectively(Coates, 2000). The bands of 1785 and 1678 cm^{-1} can be attributed to the stretching vibrations of ester C=O and C=C of corn oil (Guillén and Cabo, 1998). The bands at 1265 cm^{-1} could be assigned to the C-O stretch (Alexa et al., 2009).

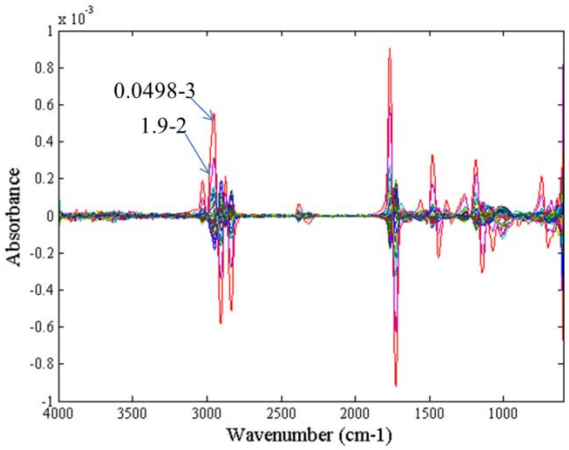

Figure 5.1 First derivative-mean centered spectra of ZON-contaminated corn oil

5.3.2 Characterization of ZON in corn oil

PCA analysis was applied for identifying the specific bands of ZON, excluding the outlier samples, and investigating the possibility of detection of ZON in corn oil by FTIR and chemometric models. PCA was performed by using the calibration set with 45 samples and the preprocessed methods of first derivative (order:1, points: 29)-mean center. From the Hotelling T^2, it was found 0.0498-3 was significantly different with other samples and influenced the Q residuals suggesting it was an outlier and should be removed out. Besides, 1.6-1, 1.6-3, 1.9-1, 1.9-2 and 0.0498-1 also showed distinct difference or high Q residuals, and may be the cause of error. From the score plot of PC1 and PC2, it was noticed that 0.0498-3 and 1.9-2 was significantly different with others, and was out of the 95% confidence range, confirming they are the outliers, which has the consistent result observed from Figure 5.2. From Figure 5.2(b), it was also found that the samples were generally separated into two groups according to the concentration level of ZON. The samples with higher level were located on the negative side of PC1 and the samples with lower concentration were on the positive side. From

the loading plot of PC1, distinct differences related to the classification were at 3071-2766 cm^{-1}(A), 1830-1661 cm^{-1}(B), 1661-1277 cm^{-1}(C), 1277-883 cm^{-1}(D) and 883-600 cm^{-1}(E). Because the spectral variation in region E was influenced with the error caused by the IR instrumentation, the region E was removed out for the following quantitative analysis.

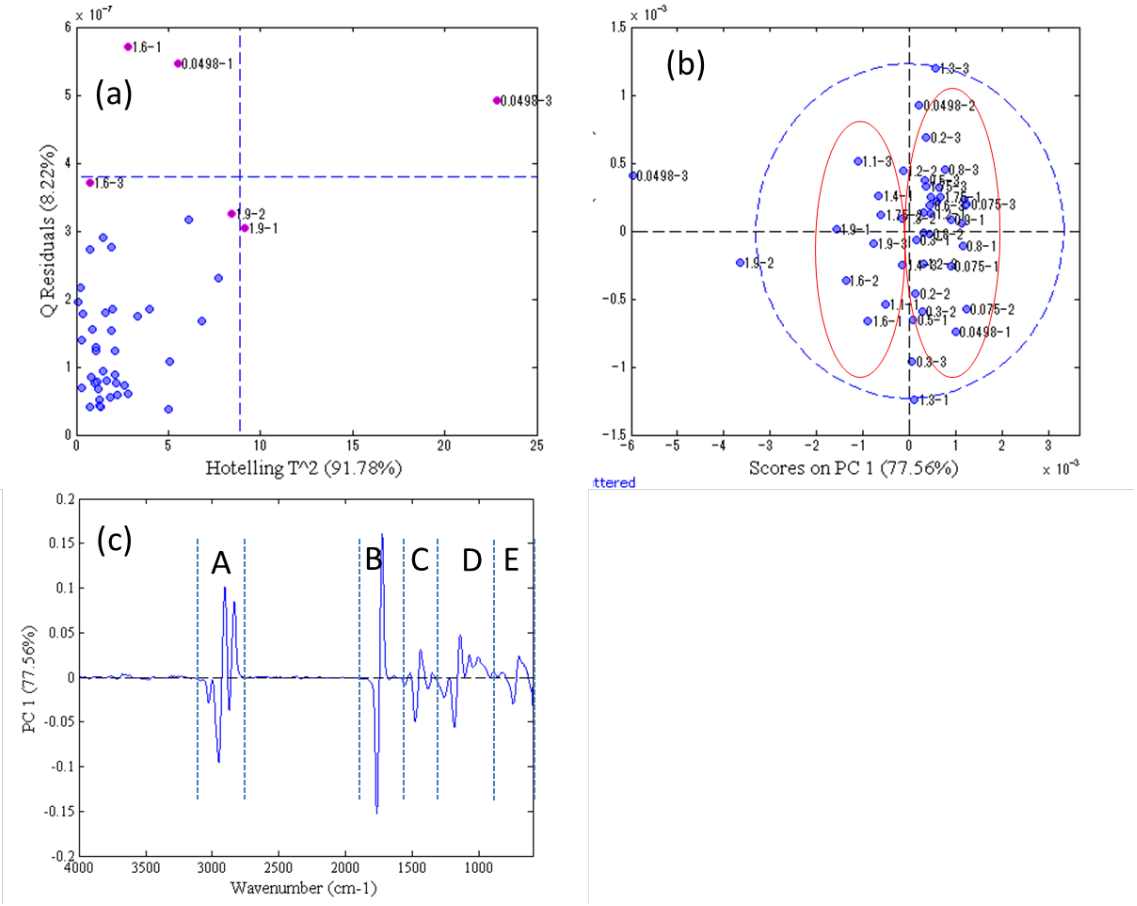

Figure 5.2 The plots of Hotelling T^2 vs Q residuals (a), PC1 vs PC2 scores (b) and PC1 loadings (c).

5.3.3 Spectral region selection for ZON in corn oil

The quantitative analysis was conducted by using PLS with the combination of the spectral regions selected above. The models were firstly developed with the preprocessing method of first derivative-mean center. Table 5.1 shows the results of PLS with different spectral regions. It can be found that PLS with the region of ABCD presents the best cross-validation results, with R^2_{CV} of 0.789, and RMSECV of 0.267,

while PLS with the region of BCD (1830-883 cm^{-1}) presents comparable results with that of ABCD, but produced better predictions. Thus, the PLS model with region of BCD was further investigated. The detailed information on the model with the region of BCD was shown in Figure 5.4(a). Variable importance in projection (VIP) is a measure to accumulate the importance of each variable reflected by loading weights from each component. The VIP measure v_j is defined as

$$v_j = \sqrt{p \sum_{a=1}^{A} \left[SS_a (w_{aj}/\|w_a\|^2)\right] / \sum_{a=1}^{A} (SS_a)} \qquad (5.1)$$

Where SS_a is the sum of squares explained by the a_{th} component. $(w_{aj}/\|w_a\|^2)$ represents the importance of the j_{th} variable.

Generally, a proper threshold=1 yield more relevant variables (Mehmood et al. 2012). Figure 5.3(a) shows the VIP scores varied with the wavenumber 1830-883 cm^{-1}. It was shown that the bands at 1745-1724, 1668 and 1169 cm^{-1} included the most relevant variables for PLSR.

The region of 1745-1724 cm^{-1} was related to ester C=O vibration of corn oil, and the bands of 1668 and 1169 cm^{-1} corresponded to the vibrations of C=C and C-O of corn oil (Guillén and Cabo, 1998), This result was consistent with that of Regression vector (Figure 5.3(b)), which showed that the bands at 1830-1600 cm^{-1} and 1280-1050 cm^{-1} contributed the most to the regression model, while the 1380-1280 cm^{-1} region had less influence on the model. Hence, the spectral region at 1380-1280 cm^{-1} was removed out. However, the model was not improved after removing the band of 1380-1280 cm^{-1} (Figure 5.4(b)). To further improve the PLS model, a variable selection method iPLS was applied in the range of 1830-883 cm^{-1}. ~20 intervals with 20 points in each interval were selected for model development. The results in Table 5.1 (row 11-12) and Figure 5.5a presented that the regions of 1830-1745, 1685-1600, 1425-1253, 1164-1108, 1049-964, 933-877 cm^{-1} could generate the optimal PLS model. The cross validation was superior, with RMSECV of 0.247 and R_{CV}^2 of 0.820, but the prediction result was far inferior to that of PLS based on the region of BCD.

Table 5.1 Comparison of region combination for PLS model

Model	Region	R^2_{CV}	RMSECV	RMSEP
PLS	all	0.625	0.356	0.478
	ABCD	0.789	0.267	0.316
	ABC	0.594	0.365	0.496
	BCD	**0.776**	**0.271**	**0.294**
	ABD	0.712	0.302	0.348
	ACD	0.584	0.378	0.489
	AB	0.577	0.372	0.513
	BC	0.647	0.340	0.427
	CD	0.512	0.389	0.467
	AD	0.496	0.451	0.589
iPLS	1830-1745, 1685-1600, 1425-1253, 1164-1108, 1049-964, 933-877	0.820	0.247	0.424

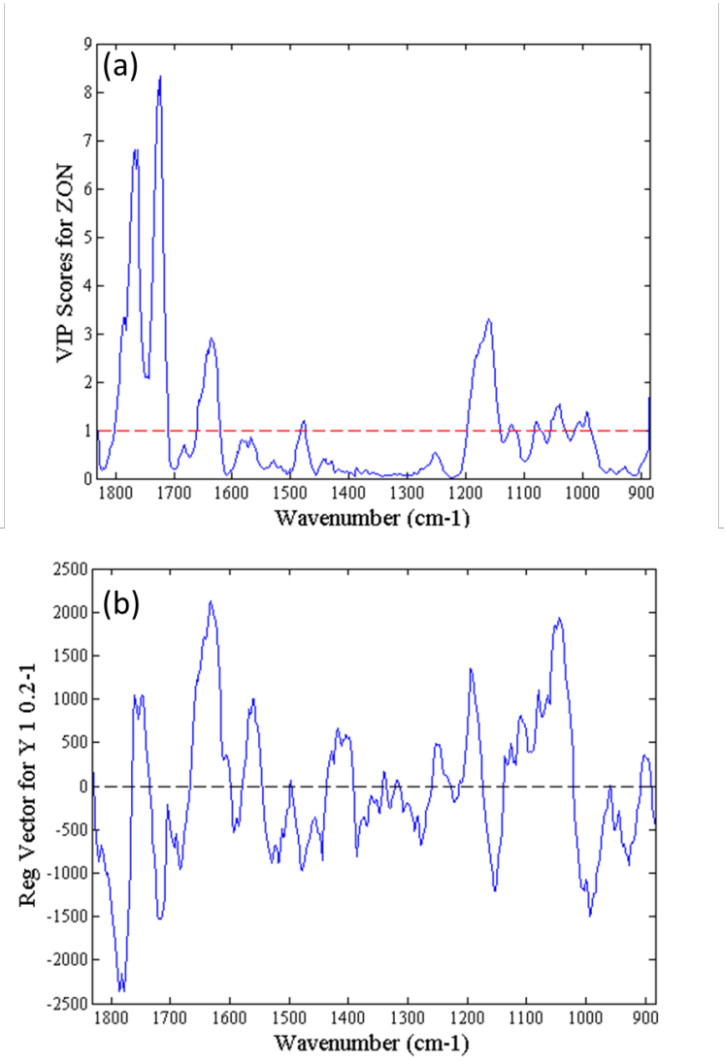

Figure 5.3 VIP score and Regression vector for PLSR

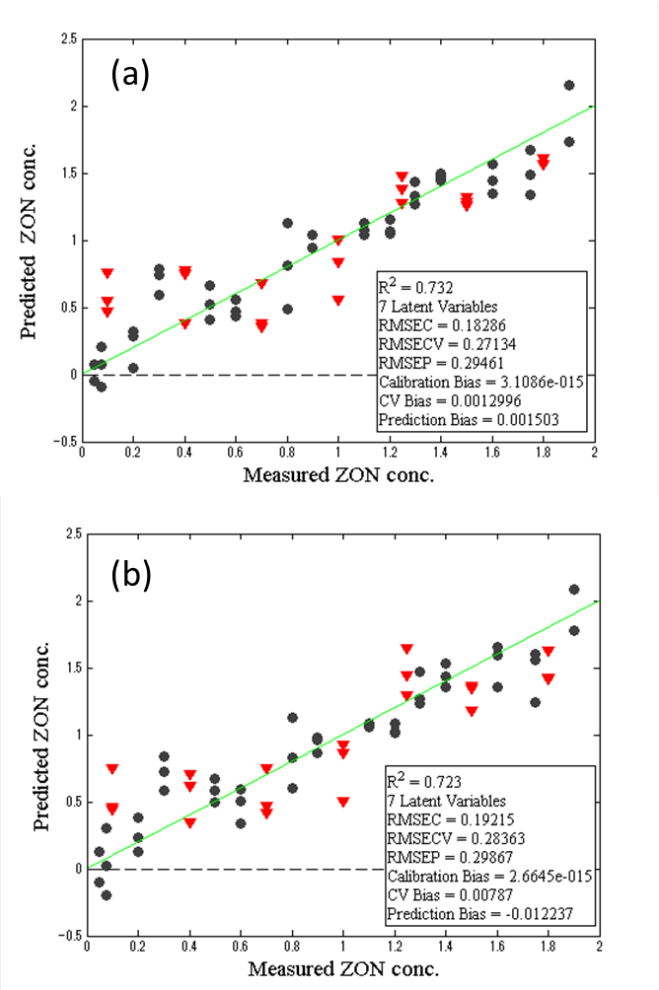

Figure 5.4 PLS regression line with the spectral region: (a) 1830-883 cm^{-1} (b) 1830-1380 cm^{-1} and 1280-883 cm^{-1}.

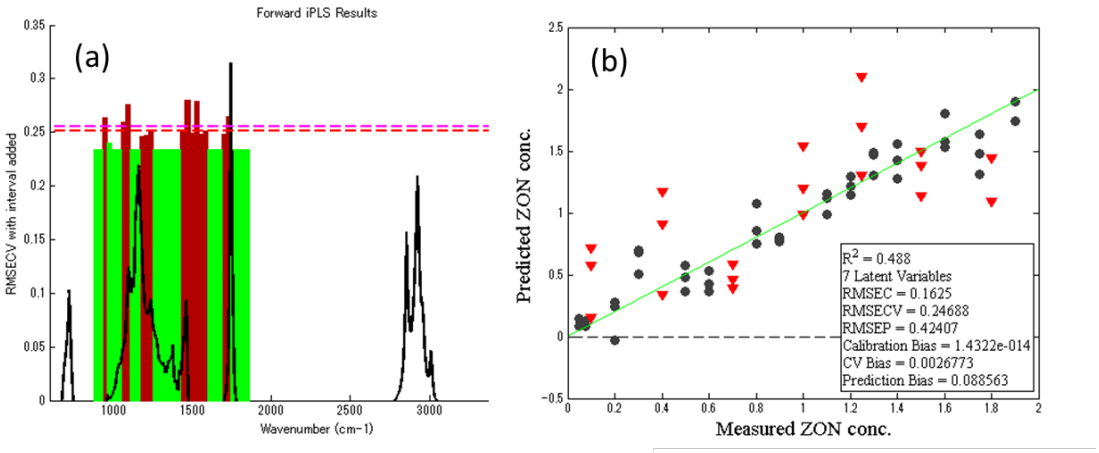

Figure 5.5 Selected intervals (a) and prediction result (b) from iPLS

Table 5.2 Comparison of region combination for PCR model

Model	Region	R^2_{CV}	RMSECV	RMSEP
	ABCD	0.787	0.265	0.324
	ABC	0.606	0.361	0.405
	BCD	**0.758**	**0.282**	**0.313**
	ABD	0.710	0.301	0.352
PCR	ACD	0.596	0.385	0.469
	AB	0.583	0.401	0.432
	BC	0.563	0.436	0.479
	CD	0.463	0.503	0.526
	AD	0.488	0.516	0.519
iPCR	1830-1417,1377-877	0.775	0.272	0.328

To compare with PLS, PCR was performed by using different combinations of the spectral regions A, B, C and D. The best prediction of PCR was obtained by selecting the same regions with that of PLS, confirming that BCD region was specific for ZON and contributed most to the modeling development (Table 5.2). iPCR was performed for improving the prediction of the PCR model (Table 5.2). However, identical with that of iPLS, the cross-validation was superior to that of PCR with the region of BCD, but the prediction error and correlation coefficient showed poorer results. The results of iPLS and iPCR revealed that the selected regions were more specific for calibration set than validation set, which was called over-fitting for the validation. Therefore, these spectral

selection methods were not suitable for improving the performance of the models and selecting the specific regions for ZON. Finally, the region of BCD was the most specific for prediction of ZON in corn oil. The selection of this region may be caused by the characteristics of the ZON standard IR absorption and the molecules interaction between ZON and corn oil. As seen in the IR spectrum of ZON in Figure 3.2, the IR bands of ZON were strongly concentrated in the range of 1800-600 cm^{-1}, and most of these bands corresponded to the aromatic ring of ZON. Thus, it has the possibility that the aromatic ring of ZON has strong interaction with the composition of corn oil, which results into the successive variation of IR absorption related to the ZON concentration in the region of 1830-883 cm^{-1}.

5.4 Conclusions

The PLS and PCR for detection of ZON in corn oil were optimized by using spectral region selection methods.

The spectral regions for PLS (1830-883 cm^{-1}) were initially selected by PCA. The VIP scores, regression vector and IPLS were performed for further extracting the specific regions for better prediction. However, the spectral regions by VIP, Regression vector did not improve the model, compared to the PLS model with the region selected by PCA. Compared to the PLS with 1830-883 cm^{-1} region, the PLS with the spectral regions by iPLS produced better cross validation result, but inferior prediction. As a comparison to PLS and iPLS, PCR and iPCR were applied in the 1830-883 cm^{-1} region. Compared to PCR, iPCR produced better cross validation result, but inferior prediction, which was identical to the comparison between PLS and iPLS. The inferior predictions of iPLS and iPCR confirmed that the spectral region of 1830-883cm^{-1} was specific for ZON in corn oil. The optimum PLS model in the range of 1830-883 cm^{-1} had R_p^2 of 0.732, and RMSEP of 0.299. Further investigation is necessary to develop PLS model for ZON in corn oil with bigger dataset.

CHAPTER VI
SIMULTANEOUS DETERMINATION OF 15-ACETYLDEOXYNIVALENOL AND ZEARALENONE IN CORN OIL BY ATR-FTIR SPECTROSCOPY

6.1 Introduction

15-acetyldeoxynivalenol (15-AcDON) and zearalenone (ZON) are second derivatives of the fungi *Fusarium spp.*, mainly present in cereal and cereal-based foods. Attention was raised on these mycotoxins, because the presence of 15-AcDON in food is associated with swine feed refusal and vomiting, and the ingestion of ZON can cause serious breeding problems, such as infertility, abortion to human beings and domestic animals. Moreover, produced by the same group of toxigenic fungi, 15-AcDON and ZON are found simultaneously in corn (Hsia et al., 1987), wheat (Perkowski et al., 1990) and oats (Müller et al., 1988). Recently, researchers and organizations paid more attention on the occurrence of mycotoxins in edible oil (Schollenberger et al., 2008; Li et al., 2014; Bordin et al., 2014). It has been found that some mycotoxins, such as trichothencenes, ZON, contaminated edible oil with high levels. Specifically, ZON was the most present mycotoxin in edible oil, and 15-AcDON has higher contamination level compared to other trichothecenes (Schollenberger et al., 2008; Bordin et al., 2014). Furthermore, a survey conducted in China showed that several mycotoxins co-occurred in edible oil (Li et al., 2014).

The multi-mycotoxin methods, allowing to simultaneously determining several mycotoxins, have been developed by using HPLC-MS/MS and GC-MS (Berthiller et al., 2007; Tanaka et al., 2000). These methods are reliable and precise, but extraction and cleanup are required before instrumentation analysis, which are time-consuming and environmentally unfriendly. Besides, the operation of HPLC-MS/MS or GC-MS needs a high level of expertise and experience.

Mid-infrared spectroscopy (MIR) is a rapid method with very few operations and no sample extraction. More recently, as a direct and reliable method, Fourier transform infrared (FTIR) spectroscopy makes it possible to simultaneously obtain specific information about different parameters of samples, mainly in the 4000–600 cm^{-1} region (Abramovic et al., 2007). In recent years, FTIR spectroscopy has been applied for the

simultaneous determination of the compositions or contaminants in foodstuffs (Bureau et al., 2009; Ahmadi, and Arshadi, 1999). However, the application of FTIR spectroscopy for simultaneous determination of several mycotoxins has not been reported. So the objective of this study is to develop a simple, fast and environmentally friendly FTIR spectroscopy-based procedure for 15-AcDON and ZON determination in refined corn oil.

6.2 Materials and methods

6.2.1 Sample preparation

15-AcDON standard and corn oil were purchased from Wako Pure Chemical Industries, Ltd. Japan. ZON was purchased from Sigma-Aldrich (Sigma Chemical, St. Louis, M.O.U.S.A.).

Stock solution of 15-AcDON and ZON: 15-AcDON or ZON in pure crystalline form was dissolved in pure methanol to prepare 100ppm 15-AcDON-methanol or ZON-methanol solution.

Artificial contamination of 15-AcDON or ZON in corn oil: 100 ppm 15-AcDON-contaminated corn oil and 10ppm ZON-contaminated corn oil were firstly prepared. The procedures were as follows: add the stock solution into corn oil, followed by removing the residual methanol of corn oil in a desiccator with reduced pressure.

For simulation of the actual co-contamination of 15-AcDON and ZON in corn oil, the artificially contaminated corn oil samples were prepared by diluting and mixing the above resulted contaminated samples

6.2.2 FTIR spectroscopy measurement

IR Spectra were collected using MB3000 FT-IR spectrometer (ABB Inc. Canada) with a MIRacle ZnSe45° attenuated total reflectance (ATR) accessory (PIKE Tech. USA) in the 4000-600 cm^{-1} region. The absorbance of samples was measured at a resolution of 4 cm^{-1} and with 32 scans. Air-conditioner in the laboratory turned on to keep a stable room temperature of 25℃ and a steady level of humidity.

For the corn oil contaminated with 15-AcDON and ZON, the absorbance spectrum was collected against a background obtained with a dry and empty ATR cell. Before recording each spectrum, the ATR crystal was cleaned with a KimWipe tissue

(Kimberly-Clark Corporation, USA) and 70% ethanol solution to minimize sampling error, the mean of the three spectra were collected from the same corn oil sample for the following analysis step.

6.2.3 Preprocessing methods

Various filtering and normalization methods were tried for optimizing the PLS models with full spectral regions. For 15-AcDON, the combination of Baseline correction (order:1)-Standard Normal Variate (SNV)-mean center was used. For ZON, the combination of First Derivative (order:2, window: 29 points)-Smoothing (order:0, window: 5 points)-Mean center was performed for the PLS models.

6.2.4 Selection of spectral regions

Three methods were used to select informative spectral regions: genetic algorithm-Partial Least Square (GA-PLS) and interval Partial Least Square (iPLS). The informative spectral regions were determined by referring to the lowest root mean square errors of cross-validation (RMSECV).

Before GA-PLS and iPLS, the spectral regions with much noise and high interference were firstly identified and removed by PRVS (Preprocessing plot, Regression Vector, VIP Scores and Selectivity Ratio), and the remained regions were selected for further optimization of 15-AcDON and ZON analysis by GA-PLS and iPLS. The optimal parameters of GA-PLS and iPLS were selected according to the lowest RMSECV.

For 15-AcDON, the parameters of GA-PLS were: population size: 64, window width: 3, initial terms: 30, max generations: 100, percentage at convergence: 50%, mutation rate: 0.005, cross-validation: random with 10 latent variables(LV), 5 splits and 1 iteration, replicate run :1. The parameters of iPLS were: Number of intervals: ~20, interval size: 3 variables, Max LV: 15.

For ZON, the parameters of GA-PLS were population size: 64, window width: 10, initial terms: 30, max generations: 100, percentage at convergence: 50%, mutation rate: 0.005, cross-validation: random with 10 latent variables (LV), 5 splits and 1 iteration, replicate run :1. The parameters of iPLS were Number of intervals: ~20, interval size: 10 variables, Max LV: 15.

The GA-PLS and iPLS were carried out by PLS_Toolbox (Eigenvector Research Inc., WA, USA) in MATLAB software (The MathWorks Japan Inc., Tokyo, Japan)

6.2.5 Statistical analysis

The abnormal spectra were evaluated by Q residuals (confidence limit of 95%) and Hotelling's T^2. The Q residuals indicate the magnitude of the variation remaining in each sample after projection through the model, whereas the Hotelling's T2 represent how far each sample is from the center of the model. The calibration and validation procedures were performed using PLS_Toolbox in MATLAB. The total 69 spectral data were automatically split into calibration and validation set by Kennard-Stone method. Kennard-Stone has the advantage of keeping unique samples which best fill out all covariance space. Calibrations of partial least squares (PLS) were performed by using 70% of the total spectra, and validations were performed by the other 30% of the total spectra to avoid over-fitting the PLSR equations.

The performance of the developed models were evaluated by RMSEC (root mean square error of the calibration), RMSECV (root mean square error of the cross-validation), RMSEP (root mean square error of the prediction), R^2 (coefficient of determination).

6.3 Results and discussion

6.3.1 Corn oil samples artificially contaminated with 15-AcDON and ZON

A total of 69 corn oil samples with 15-AcDON and ZON at 23 levels were prepared by diluting the 100ppm 15-AcDON and 10ppm ZON contaminated corn oils with pure corn oil, followed by mixing the dilutions. As shown in Table 6.1, the concentration ranges of 15-AcDON and ZON were at 0.1-2.25 ppm and 0.075-1.9 ppm, respectively. The concentration ranges of 15-AcDON and ZON were designed by considering the actual contamination conditions in edible oil. The contamination level of 15-AcDON and ZON in edible oil was related to the toxigenic *Fusarium* spp. of ram materials and the refining process. It has been found that 15-AcDON and ZON were produced by different kinds of *Fusarium spp*. in cereals. 15-AcDON was produced by *F. graminearum*, while ZON was the main derivative of *F. equiseti, F. semitectum, F. oxysporum, F. moniliforme, and F. equiseti* (El-Kady & El-Maraghy, et al. 1982; Ezekiel, et al. 2008). In addition, temperature and water activity have significant influence on the production of mycotoxin

by the *Fusarium spp.(* Kokkonen, et al. 2014). Usually, several *Fuasrium spp.* occurred simultaneously in cereals (Gordon, 1952). Thus, the kinds of Fusarium spp. and the influence of temperature and water make it difficult to characterize the contamination correlation between 15-AcDON and ZON. The recent studies have also showed the inconsistent results on the correlation of 15-AcDON and ZON in actual contamination of cereals (Magan, et al. 2002). Moreover, because different oil refining process would result in the different elimination effect to different kinds of mycotoxins, the finial concentration of 15-AcDON and ZON in edible oil would vary (Schollenberger et al., 2008; Kamimura et al., 1986). Therefore, the concentration ratio of 15-AcDON and ZON was prepared randomly in corn oil samples.

Table 6.1 Concentration data for the co-contamination of ZON and 15-AcDON in corn oil

Sample No.	Concentration (ppm)	
	ZON	15-AcON
1	0.075	0.15
2	0.1	0.5
3	0.15	0.75
4	0.2	0.8
5	0.3	0.4
6	0.35	2.1
7	0.4	0.35
8	0.5	0.6
9	0.55	1.1
10	0.6	0.9
11	0.7	0.55
12	0.875	1.75
13	0.9	1.3
14	1	2.25
15	1.125	1
16	1.2	0.2
17	1.3	0.95
18	1.5	1.2
19	1.6	1.8
20	1.75	0.3
21	1.9	1.6
22	0.75	0.1
23	0.18	0.4

6.3.2 Characteristics of the IR spectra

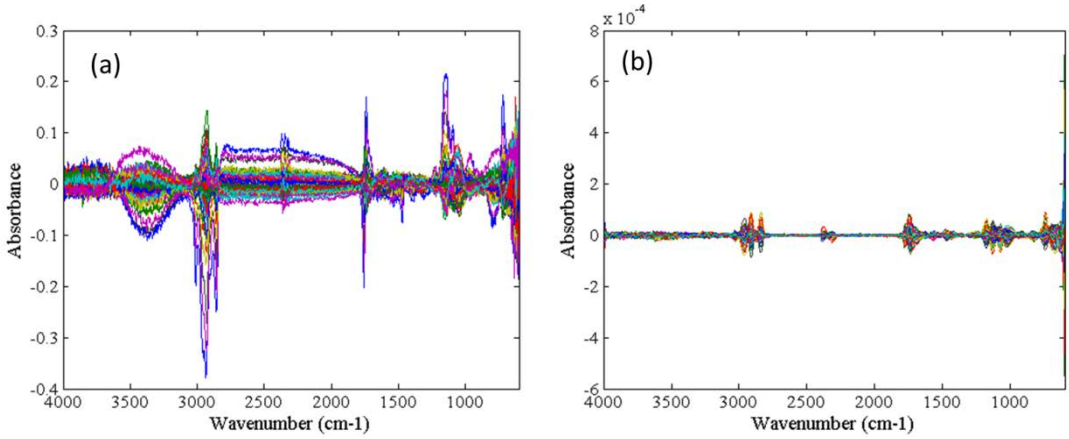

Figure 6.1 Infrared spectra of contaminated corn oil with different preprocessing methods: (a). after C1; (b). after C2.

In order to extract the informative bands for 15-AcDON and ZON for the PLS models, two combinations of spectral preprocessing methods were used: Combination 1 (C1): baseline correction (order:1), SNV and mean center; Combination 2 (C2): First Derivative (order:1, window width: 29 points)-Smoothing (order:0, window width: 5 points)-mean center. For developing PLS models within full spectral region, C1 was the optimal for 15-AcDON analysis, and C2 was used for prediction of ZON in corn oil. Figure 6.1a and Figure 6.1b present the IR spectra with C1 and C2, respectively. For both spectra, big noise observed at around 700-600 cm^{-1} was caused by the FTIR spectrometer. Both spectra showed IR band difference at 3100-2700 cm^{-1}, 1800-1600 cm^{-1}, but the IR band difference at about 2422-2272 cm^{-1} was resulted from the carbon dioxide. After C1, the baseline was corrected and the scatter was reduced. In addition, no IR absorption was observed in the range of 4000-3755 cm^{-1}, and the broad band ranged 3755-3100 cm^{-1} was probably caused by the O-H stretch of the water in the oil samples. Obvious scatter was observed in the range of 2700-1900 cm^{-1}, which was probably caused by the OH stretch of water of the oil samples (Kos, et al. 2004). After C2, the broad band and scatter caused by water in Figure 6.1a was corrected, and no IR absorption difference was found in the regions of 4000-3100 cm^{-1}, and 2700-1900 cm^{-1}.

In Chapter III, it was found that the specific bands of low levels of 15-AcDON and ZON were located at 1090-1075 cm^{-1} and 1760-1730 cm^{-1}, respectively, revealing that no IR bands overlapping and interference between 15-AcDON and ZON. However, Chapter IV and V found that 1901-881 cm^{-1} and 1830-883 cm^{-1} contributed to PLS predictions for 15-AcDON and ZON, respectively, suggesting that 15-AcDON and ZON of corn oil had overlapping bands with PLS models. The results of Chapter III and Chapter IV and V are reasonable because the low concentration levels of the mycotoxins could be easily influenced by the fatty acids and esters of the corn oil, and therefore the accuracy of the prediction of the PLS models can be improved by selecting as more as specific and informative bands. In addition, the difference of sample numbers and concentration range of Chapter III and Chapter IV and V might also influence on the specific band ranges. Hence, the mycotoxin prediction of the PLS models was based on the synthetic action between compositions of corn oil and mycotoxins molecules, rather than one or two IR bands of mycotoxins molecules, and the IR overlapping bands between 15-AcDON and ZON might interfere the prediction of 15-AcDON and ZON in corn oil by PLS models.

6.3.3 Optimization of the PLS models of 15-AcDON and ZON in corn oil by the use of optimized spectral regions based on PRVS, GA-PLS and iPLS

In this study, the PLS models with the full spectral regions were firstly performed for calibration of 15-AcDON and ZON. The spectral regions were initially selected or removed according to the preprocessing plots in Figure 6.1, Variable Importance of Projection (VIP) scores, Regression vector and Sensitivity Vector (PRVS). Specifically, the spectral regions with VIP scores lower than 1 indicated that the IR absorption of this region did not significantly contribute the models (Mehmood et al. 2012). The Sensitivity Vector suggested that whether the selected regions have sensitive response to the PLS models (Mehmood et al. 2012). After PRVS, GA-PLS and iPLS were performed and compared for further models optimization.

6.3.3.1 15-AcDON in corn oil

The PLS model with full spectral region was firstly performed. As shown in Table 6.2, the cross validation result was R^2_{CV} of 0.455 and RMSECV of 0.499. In Figure 6.2,

the VIP scores and Regression vector of PLS model present high contribution loadings at around 700-600 cm^{-1}, which was caused by the noise from the FTIR spectrometer and should be removed, according to the analysis of 6.2.1. The region of 4000-3755 cm^{-1} was removed, because no IR absorption was observed in the region, which was confirmed in the selectivity Ratio (zero with much noise) and VIP scores (less than 1). According to the Selectivity Ratio and 6.2.1, the regions of 3755-3196 cm^{-1} and 2820-1825 cm^{-1} were removed because the scatter in these regions was caused by the OH stretch of water in oil and significantly deteriorated the prediction of PLS model (Kos, et al. 2004). In addition, the regions of 1690-1309 and 980-700 cm^{-1} were removed because the VIP scores v_j of the regions were lower than 1. Hence, the regions of 3197-2821 cm^{-1}, 1824-1703 cm^{-1}, and 1211-981 cm^{-1} were initially selected. Because this region selection method was based on the preprocessing method, Regression Vector, VIP Scores and Selectivity Ratio, this region selection method was called PRVS. The result of PRVS displayed in Table 6.2 shows that the RMSECV of 0.472 was lower than that of the full range.

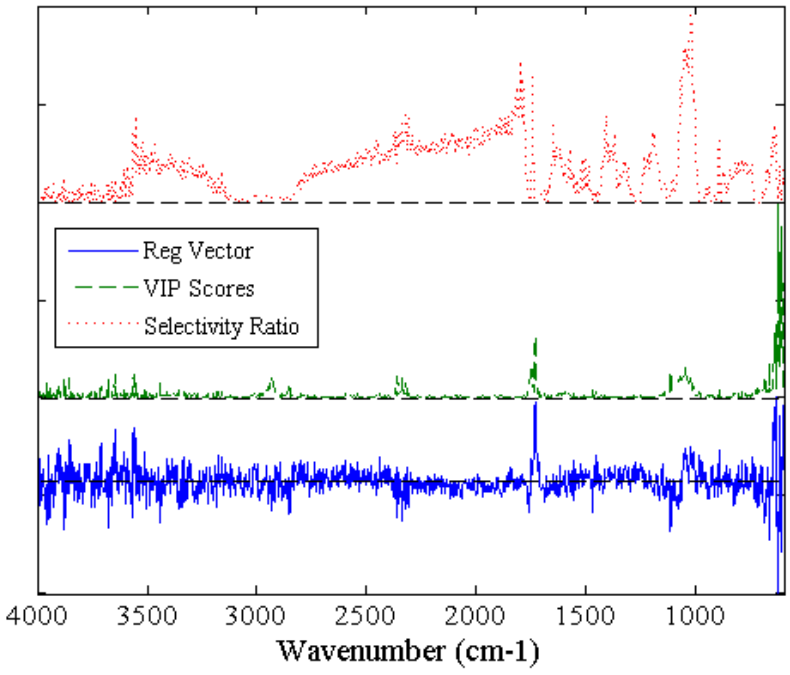

Figure 6.2 Regression vector, VIP scores and Selectivity Ratio of the PLS model for 15-AcDON in corn oil

iPLS and GA-PLS were performed in the regions obtained from PRVS. The parameters of the iPLS and GA-PLS methods were compared for selecting the best PLS models. For iPLS, the number of intervals was selected from 1 to 20, and the variable numbers of each interval were ranged 2-20. 20 was chosen as the Max LV numbers of the PLS models. It was found that 16 intervals and 3 variables of each interval could generate the best result. As shown in Table 6.2 (row 3-7), the values of R^2_{CV} and RMSECV were 0.805 and 0.307, respectively.

For GA-PLS, it was found that population size and window width (variable numbers) significantly influenced the results. Basically, with bigger population size and fewer variables of each window, the RMSECV would be smaller, but the calculation time would be longer. On the other hand, the RMSECV could not be distinctly improved, when the population size was higher than 64, and the variables were lower than 3. Hence, the population size and variable numbers of each window were 64 and 3, respectively. Random subset with 5 splits and 1 iteration was chosen as the cross validation method. The result of GA-PLS was shown in Table 6.2 (row 9-14). The values of R^2_{CV} of 0.716 and RMSECV of 0.358 were superior to that of PRVS, but inferior to that of iPLS. Compared the regions of GA-PLS and iPLS, it can be found that the obvious region difference was that GA-PLS has regions of 3149-2900 cm^{-1}, while iPLS has regions ranged 1784-1768 cm^{-1}. The 3149-2900 cm^{-1} region corresponded to the O-H stretch of corn oil, and the 1784-1768 cm^{-1} might be caused by the ester C=O stretch of corn oil, suggesting that the 1784-1768 cm^{-1} band of ester C=O in corn oil may be related to the variation of 15-AcDON concentrations.

Table 6.2 Cross validation results of spectral region selection methods for 15-AcDON in corn oil

Method	Spectral region (cm^{-1})	LV No.	R^2_{CV}	RMSECV
PLS	All	7	0.455	0.499
PRVS[a]	3197-2821, 1824-1703, 1211-981	6	0.508	0.472
iPLS	2894-2891, 2877-2873, 2842-2839, 2831-2827, 1784-1780, 1772-1768, 1737-1733, 1703-1699, 1193-1184, 1170-1155, 1147-1143, 1135-1126, 1008-999, 991-981	15	0.805	0.307
GA-PLS	3149-3132, 3120-3112, 3024-3016, 2927-2920, 2908-2900, 2879-2871, 2840-2833, 2821-1818, 1739-1731, 1191-1174, 1153-1135, 1124-1116, 1095-1087, 1076-1068, 1027-1020, 1008-991	11	0.716	0.358

a: PRVS represented the Preprocessing plot, Regression Vector, VIP Scores and Selectivity Ratio.

On the other hand, the cross-validation results of iPLS and GA-PLS have the potentials for screening 15-AcDON in corn oil. One of the disadvantages of iPLS and GA-PLS was over fitting of the calibration models, which refers to the poor prediction results based on the calibration models of iPLS and GA-PLS. In order to confirm the high performance of the optimized PLS models based on iPLS and further compare the prediction results of GA-PLS and iPLS, the total spectra were separated into calibration and validation dataset with a ratio of 70: 30 by Kennard-Stone method. The prediction of iPLS regression had R^2_p of 0.784 and RMSEP 0.293, superior to that of GA-PLS model with R^2_p of 0.753 and RMSEP 0.312, confirming the better performance of GA-PLS and ATR-FTIR spectroscopy to determine 15-AcDON in corn oil.

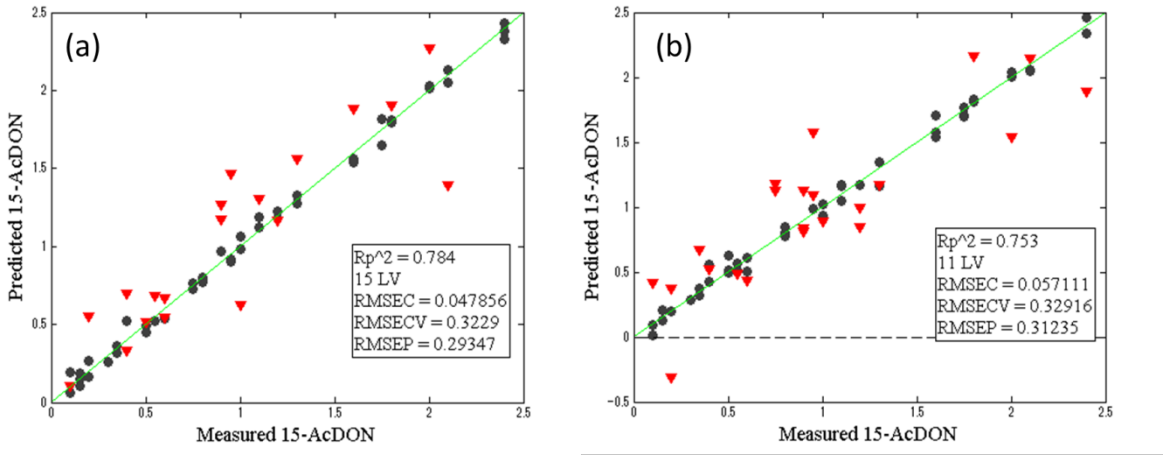

Figure 6.3 IPLS (a) and GA-PLS (b) regressions for prediction of 15-AcDON in corn oil

6.3.3.2 ZON in corn oil

For ZON in corn oil, the spectral region was selected according to the preprocessing plot of Figure 6.1b and the VIP scores of Figure 6.4. The Regression vector and Selectivity Ratio showed that spectra with high frequency were difficult for interpretation. The spectral regions of 1818-1546 cm^{-1} and 1247-904 cm^{-1} were initially selected, and the selected regions improved the performance of the PLS model, as shown in Table 6.3. To further optimize the PLS model, the regions of 1818-1546 cm^{-1} and 1247-904 cm^{-1} were utilized as the original regions for the informative regions by using GA-PLS and iPLS in the development of PLS regression models for ZON in corn oil.

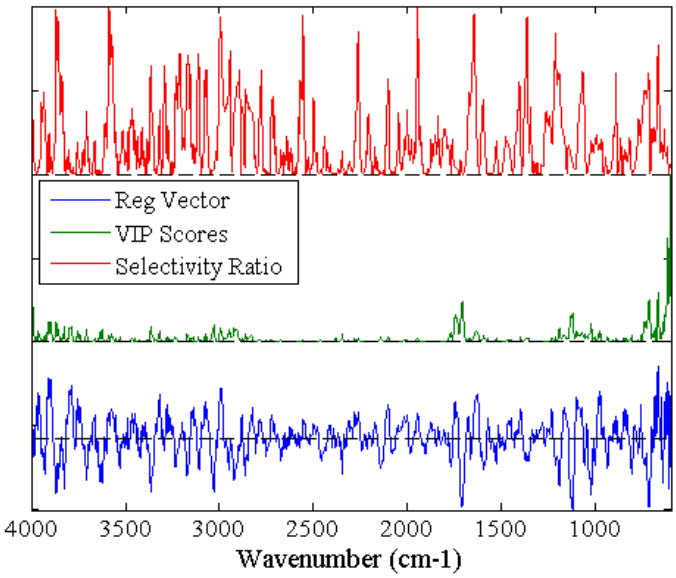

Figure 6.4 Regression vector, VIP scores and Selectivity Ratio of the PLS model for ZON in corn oil

Table 6.3 Cross validation results of spectral region selection methods for ZON in corn oil

Method	Spectral region (cm-1)	LV No.	R^2_{CV}	RMSECV
PLS	all	9	0.126	0.568
PRVS[a]	1818-1546,1247-904	9	0.481	0.410
iPLS	1820-1533, 1261-1243, 1222-1205, 1164-935, 914-904	9	0.476	0.414
GA-PLS	1816-1809, 1797-1789, 1739-1722 1710-1703, 1681-1674, 1623-1616 1595-1577, 1247-1240, 1218 -1201 1054-1047, 1026-1018, 939-912	9	0.501	0.401

a: PRVS refers to the Preprocessing plot, Regression Vector, VIP Scores and Selectivity Ratio.

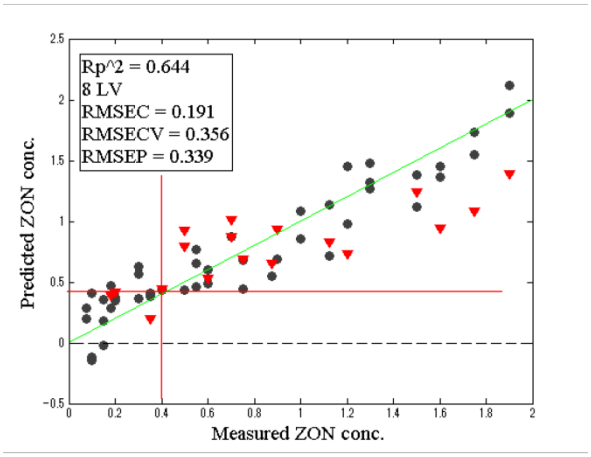

Figure 6.5 GA-PLS regression for prediction of ZON in corn oil

The informative regions were used to develop the GA-PLS and iPLS models. Compared to iPLS, the GA-PLS model gives better cross validation results, with higher R^2_{CV} and lower RMSECV (Table 6.3). The robustness of the GA-PLS model with cross-validation for ZON in corn oil was also checked by the use of 30% of the total spectra as validation dataset. As demonstrated in Figure 6.5, the GA-PLS model with validation had R^2_p of 0.644 and RMSEP of 0.339, suggesting that the PLS model was not adequate for quantitatively determining ZON in corn oil. However, the model with R^2_p of 0.644 may allow discriminating the samples with low and high levels of ZON in corn oil. In order to evaluate the classification ability of the model, a threshold of 0.4 ppm ZON was set according to the maximum allowed value of European commission (Commission Regulation (EC) No 1881/2006 of 19), and lines corresponding to target threshold (0.4 ppm ZON) are added in the GA-PLS calibration and prediction plot (Figure 6.5). They allow to visualizing easily if there are false positives and/or false negatives (by positive we mean contaminated above 0.4 ppm), if predicted concentration level agree with measured one or if some sample is not fitted in the right group. As shown in Figure 6.5, the model allowed the separation of almost all the samples with ZON content <0.4ppm from those with a contamination >0.4ppm, although 3 samples were classified into the false positive region incorrectly.

Until now, to our knowledge, the simultaneous determination of several mycotoxins by IR/NIR spectroscopy has not been reported. But the investigations have been made on simultaneous determination of other contaminants in food, such as pesticide. Ahmadi

and Arshadi (1999) determined the pesticides of naptalam, N-(1-napththyl)phthalamic acid (NAP), and its metabolites, 1-naphthylamine (NNA) and N-(1-naphthyl) phthalimide (NPhDA), in natural water by FTIR spectroscopy. The result showed high recoveries and low limit of detection (ppb level) for determination of the pesticides. However, the pre-concentration and methanol elution procedures were used in their study, which increased the analysis time and complexities, and was not environment-friendly. In this study, 15-AcDON and ZON in corn oil were determined by ATR-FTIR spectroscopy without any pretreatment and organic solvents. However, the PLS model prediction for ZON was only suitable for discriminating the low and high levels of ZON in corn oil. Basically, for determination of 15-AcDON and ZON in corn oil by FTIR spectroscopy, IR spectral variation should simultaneously correspond to the concentration variation of 15-AcDON and ZON in different wavenumber regions. And the IR absorption responded more accurate with higher concentration levels. However, it was noticed that the upper limit of the concentration range of ZON was 1.9 ppm, which was lower than 2.4 ppm of 15-AcDON. Thus, it is possible that the high level of 15-AcDON decreased the accuracy of determination of ZON. Therefore, future study would be conducted using the same concentration range for determination of 15-AcDON and ZON.

6.3.4 Comparison to the separate determination of 15-AcDON and ZON in corn oil

6.3.4.1 Comparison to the separate determination of 15-AcDON

In Chapter IV, the determination of 15-AcDON in corn oil was developed by GA-PLS and ATR-FTIR spectroscopy. The concentration of 15-AcDON was ranged 0.1-2.25 ppm, which was identical with this study. By comparing with the cross validation results of the GA-PLS models, the effective parameters of the optimum GA-PLS were selected as follows: population size of 128, window size of 10 variables. Determination of 15-AcDON in corn oil with another contaminant ZON in this study was compared with the separate determination of 15-AcDON in Chapter 4. Firstly, compared to the PLS models with full spectral region in both studies, it was found that the PLS model of the separate study has R^2_{CV} of 0.670 and RMSECV of 0.36, while the PLS model of 15-AcDON in this study has R^2_{CV} of 0.455 and RMSECV of 0.499, suggesting that the addition of ZON in corn oil makes the determination of 15-AcDON difficult, and effective wavenumber selection methods are

necessary for developing a multivariate model for screening 15-AcDON in corn oil.

Moreover, this study used iPLS with window size of 3 variables for determining 15-AcDON, while Chapter IV used 10 variables in GA-PLS for separately determining 15-AcDON. Because both ZON and 15-AcDON have specific IR absorption in the region of 1800-800 cm^{-1}, the determination of mycotoxins may interfere with each other in the same spectral regions. Thus, it is difficult to identify the specific IR bands varied with the concentration of the mycotoxins. On the other hand, according to the structure and IR absorptions of ZON and 15-AcDON standards, 15-AcDON has specific IR bands resulted from the functional groups of ester (O)C-O and ketone C=O, and the characteristic IR absorptions of ZON were caused by the aromatic ring and lactone bonds. So it is possible to determine ZON and 15-AcDON simultaneously with specific IR bands. However, it is difficult to identify the specific IR bands only by the spectra of 15-AcDON and ZON standards, because the IR absorptions of the mycotoxins could be influenced by the esters and fatty acids of corn oil as discussed in Chapter 3. Hence, this study carefully selected the IR bands for removing the interference bands by PRVS and iPLS. Compared to the window size (10 variables) of separate determination of 15-AcDON, the smaller window size (3 variables) in this study was reasonable, because the IR bands of ZON standard were rather intensive and consecutive to that of 15-AcDON, making the specific bands of 15-AcDON in corn oil discrete.

For determination of 15-AcDON with and without ZON in corn oil, both PLS models have acceptable prediction results. For determination of 15-AcDON in corn oil without ZON contamination, the GA-PLS has R_P^2 of 0.841 and RMSEP of 0.211, and for determination of 15-AcDON in oil with ZON, the iPLS has R_P^2 of 0.784 and RMSEP of 0.293. Compared to the separate determination, the iPLS model showed inferior prediction result, suggesting that ZON has interference on the detection of 15-AcDON in corn oil.

6.3.4.2 Comparison to the separate determination of ZON

In Chapter V, the determination of ZON in corn oil was developed by PLS in the 1800-883 cm^{-1} region. The concentration of ZON was ranged 0.0498-1.9 ppm, while the contamination level in this study was from 0.075 to 1.9 ppm. In developing the best calibration models when the lower concentration limit of ZON were used <0.075 ppm, lot

of error was observed and the PLS model was unable to predict the concentrations appropriately (results not shown) so the models were developed for the concentration ranges above 0.075ppm in this study. This found was in agreement with the earlier investigation by Dowell et al. (1999) and Petterson & Aberg (2003) for determining deoxynivalenol in wheat kernels.

Determination of ZON in corn oil with 15-AcDON in this study was compared with the individual determination of ZON in Chapter V. Firstly, compared to the PLS models with full spectral region in both studies, it was found that the PLS model of the individual determination has R^2_{CV} of 0.625 and RMSECV of 0.356, while the PLS model of ZON in this study has R^2_{CV} of 0.126 and RMSECV of 0.568, suggesting that the addition of 15-AcDON in corn oil interfered the determination of ZON.

Moreover, the investigation in Chapter V found that the iPLS and GA-PLS methods did not improve the prediction results, and the region of 1830-883 cm^{-1} produced better prediction results compared to others, which were due to the successive IR band variation with ZON concentration caused by the aromatic ring of ZON in the 1830-883 cm^{-1} region. In this study, GA-PLS with window size of 10 variables generated accept results for determining ZON in corn oil with 15-AcDION. The IR spectral regions in the models were different because both ZON and 15-AcDON have specific IR absorption in the region of 1800-800 cm^{-1}, and the addition of 15-AcDON had influence on the ZON determination. On the other hand, it is possible to determine ZON and 15-AcDON simultaneously with specific IR bands, because of the different structures and specific IR bands of 15-AcDON and ZON standards. However, due to the influence of corn oil, it is difficult to identify the specific IR bands only by the spectra of 15-AcDON and ZON standards. In this investigation, the IR bands were selected by PRVS and GA-PLS for determining ZON in corn oil with 15-AcDON. Compared to the selected 1800-883 cm^{-1} region of individual determination of ZON, the wavenumber region by GA-PLS of this study was discontinuous due to the interference of 15-AcDON in corn oil.

For prediction of ZON in corn oil with and without 15-AcDON, the PLS models have different results. For determination of ZON in corn oil without 15-AcDON contamination, the PLS in the 1800-883 cm^{-1} has R^2_P of 0.732 and RMSEP of 0.295, and for determination of ZON in oil with 15-AcDON, the GA-PLS has R^2_P of 0.644 and RMSEP of 0.339. Compared to the separate determination, the GA-PLS model showed inferior

prediction result, suggesting that 15-AcDON has interference on the detection of ZON in corn oil.

6.4 Conclusions

The feasibility of simultaneous determination of 15-AcDON and ZON in corn oil was investigated by ATR-FTIR spectroscopy and spectral region selection methods.

The spectral regions were firstly selected by PRVS (Preprocessing plot, Regression Vector, VIP Scores and Selectivity Ratio) for 15-AcDON and ZON in corn oil. The informative spectral regions for 15-AcDON and ZON in corn oil were further selected by the use of GA-PLS and iPLS. The PLS models for 15-AcDON and ZON based on GA-PLS and iPLS were found to yield better predictability, compared to the PLS models with the full spectral region. Specifically, for 15-AcDON in corn oil, iPLS model demonstrated superior performance to GA-PLS. For ZON in corn oil, significant improvement was found to the result of GA-PLS model. However, the cross-validation of GA-PLS for ZON in corn oil was still not satisfactory for prediction. The cross validations of the PLS models for 15-AcDON and ZON were evaluated by using 70% of the total spectra as calibration, and 30% of the total spectral data as validation. For 15-AcDON, the optimum prediction by iPLS had R_p^2 of 0.784 and RMSEP of 0.293. For ZON, the PLS model based on GA-PLS showed higher prediction ability with R_p^2 of 0.644 and RMSEP of 0.339, compared to iPLS, which could be used for discriminating the low from high levels of ZON in corn oil. In addition, the simultaneous and separate determination of 15-AcDON and ZON were compared in this study. It was found that both contaminants have strong interference with each other on the spectral regions and prediction results. Further study is necessary to improve PLS regression model with more data by ATR-FTIR spectroscopy.

CHAPTER VII
CONLUSIONS AND FUTURE PROBLEMS

CONSLUSIONS

In this study, the specific infrared bands of 15-AcDON and ZON with high and low concentration levels were identified and the contaminated corn oils with 15-AcDON and ZON at different concentration levels were discriminated by ATR-FTIR in combination to PCA. In addition, for prediction of 15-AcDON in corn oil, PLS, PCR and MLR regression models with different preprocessing methods and with the informative spectral regions based on GA-PLS, iPLS, iPCR and GA-MLR were developed and compared. Moreover, for prediction of ZON in corn oil, PLS and PCR regression models with the informative spectral regions based on the variable importance in projection (VIP) scores, iPLS and iPCR were developed and compared. Finally, the 15-AcDON and ZON in corn oil was simultaneously determined by ATR-FTIR spectroscopy in combination to PLS regression models with the informative spectral regions based on PRVS (Preprocessing plot, Regression Vector, VIP Scores and Selectivity Ratio), iPLS and GA-PLS.

In Chapter III, the specific bands of 15-AcDON and ZON were identified and assigned according to the discrimination results of PC scores and the loading regions. For 15-AcDON in corn oil, the 10 and 100ppm samples were discriminated according to the asymmetric stretch of C-O of 15-AcDON with IR band region at 1003-1044 cm^{-1}, while the 0, 0.1 and 1 ppm samples were separated according to the band of 1090-1075 cm^{-1}, which was attributed to the C-O stretch of corn oil caused by the variation of 15-AcDON concentration. For ZON in corn oil, it was demonstrated that the specific bands of ZON with levels higher than 10 ppm were located in the range of 1230-1150 cm^{-1}, corresponding to the aromatic ring stretch of ZON, and the classification of the 0.1, 0.5, 1.2 and 2.25ppm samples corresponded to the band of 1760-1730 cm^{-1}, resulted from the ester C=O vibration of corn oil from the variation of ZON concentration. Therefore, the specific bands of 15-AcDON and ZON with high concentration corresponded directly to the functional groups of the mycotoxins, while the specific bands of 15-AcDON and ZON with low levels were caused by the molecules interaction between the mycotoxins and compositions of corn oil.

In Chapter IV, 15-AcDON in corn oil was determined by ATR-FTIR spectroscopy

and multivariate models. The PLS, PCR and MLR models with full spectral regions were firstly compared and optimized by using different combinations of preprocessing methods. The performance of the models was further improved by using the informative spectral region selected by GA-PLS, iPLS, iPCR, GA-MLR and MLR step. In contrast to the results of multivariate models for 15-AcDON in corn oil with the full region (4000-600 cm^{-1}), the optimized informative regions or the optimized combination of the selected regions based on GA-PLS, iPLS, iPCR and GA-MLR could provide better predictability for the relevant models. In addition, the model developed using the specific regions by GA-PLS demonstrated the lower RMSECV, RMSEP and higher $R_{CV,}^2$ R_P^2, suggesting GA-PLS had superior performance to other models.

In Chapter V, ATR-FTIR spectroscopy was applied for determining ZON in corn oil. The PLS model performance was improved by using spectral region selection methods. The informative regions for ZON in corn oil were initially selected by using PCA. Then the VIP scores, regression vector and IPLS were performed successively for further extracting the specific regions to improve the PLS performance. The results demonstrated that the spectral region selected by PCA could generate better performance than the spectral regions by VIP scores, Regression vector. PLS with the spectral region by iPLS produced better cross validation result, but inferior prediction. As a comparison to PLS and iPLS, PCR and iPCR were applied in the 1830-881 cm^{-1} region. Compared to PCR, iPCR produced better cross validation result, but inferior prediction, which was identical to the comparison between PLS and iPLS. The inferior predictions of iPLS and iPCR confirmed that the spectral region of 1830-881 cm^{-1} was specific for ZON in corn oil.

In Chapter VI, 15-AcDON and ZON of corn oil were simultaneously determined by ATR-FTIR spectroscopy. The total 69 spectra data were used as calibration for selecting the informative spectral regions. The informative spectral regions for 15-AcDON and ZON in corn oil were initially selected by PRVS (Preprocessing plot, Regression Vector, VIP Scores and Selectivity Ratio), and further selected GA-PLS and iPLS according to the result of PRVS. Compared to the PLS models with the full spectral region, the PLS models for 15-AcDON and ZON based on PRVS yield better predictability. And compared to the PLS models with the spectral region by PRVS, GA-PLS and iPLS produced superior prediction ability. Specifically, for 15-AcDON in corn oil, PLS model

with the spectral region by iPLS demonstrated superior cross validation performance to that of GA-PLS. For ZON in corn oil, GA-PLS model generated higher improvement to iPLS. However, the cross-validation of GA-PLS for ZON in corn oil was still not satisfactory for prediction. The cross validations of the PLS models for 15-AcDON and ZON were evaluated by using 70% of the total spectra as calibration, and 30% of the total spectral data as validation. For 15-AcDON, the optimum prediction by iPLS had R_p^2 of 0.784 and RMSEP of 0.293. For ZON, the PLS model based on GA-PLS showed higher prediction ability with R_p^2 of 0.644 and RMSEP of 0.339, which could be used for discriminating the low from high levels of ZON in corn oil. Therefore, further study is necessary to improve PLS regression model with more data by ATR-FTIR spectroscopy. Besides, the simultaneous determination of 15-AcDON and ZON was compared to the separate determination of both mycotoxins. It was found that 15-AcDON and ZON have strong interference with each other on the wavenumber region selection and the prediction results.

The results in this study revealed that the specific bands of the mycotoxins of 15-AcDON and ZON in high and low concentration levels were caused by the mycotoxins bonds and the interaction between the mycotoxins and corn oil, respectively. From the findings of the present research, it was suggested that ATR-FTIR spectroscopy has the potential to determine not only separately but also simultaneously 15-AcDON and ZON in corn oil.

FUTURE PROBLEMS

The research of this study was not perfectly finished, owing to the limited time. Thus, further studies are necessary in the future investigation. The following recommendations are made for future study.

1. PCA in chapter III revealed that the specific bands caused by high and low levels of mycotoxins were located in different regions. But this result may not so robust because no comparison for further confirmation. In order to further interpret the existence and movement of mycotoxin in edible oil, and confirm the PCA on the specific bands regions, a data set with concentration levels from 5 to 90 ppm should be developed.
2. It was known that with bigger data set, the robustness of the prediction of the models would be improved. In order to establish multivariate models with high prediction ability, the numbers of the samples should be increased.
3. Because of the lower concentration, the IR bands of 15-AcDON and ZON in corn oil were negligible, and the IR bands caused by the interaction of mycotoxins and edible oil might be easily influenced by other parameters. Therefore, it is necessary to find suitable preprocessing methods to increase the IR bands caused by interaction of mycotoxins and edible oil, and to improve the performance of the models.
4. For determination of ZON in corn oil separately and simultaneously with 15-AcDON, the PLS models were not improved after iPLS and/or GA-PLS, compared to the PCA or PRVS. By observing the spectra, it was found that ZON generated more noise than 15-AcDON, which may result from high resolution of measurement. Therefore, the samples should be measured with resolution of 8.
5. The prediction of the selected PLS has been evaluated by R_p^2 and RMSEP, but further evaluation by other methods is necessary to test the robustness of the PLS models. The correlation coefficient between PLS and other methods could confirm the robustness of the models. In future study, an ELISA (Enzyme-linked immunosorbent assay) method would be used as the other methods.

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