Analytical Methods

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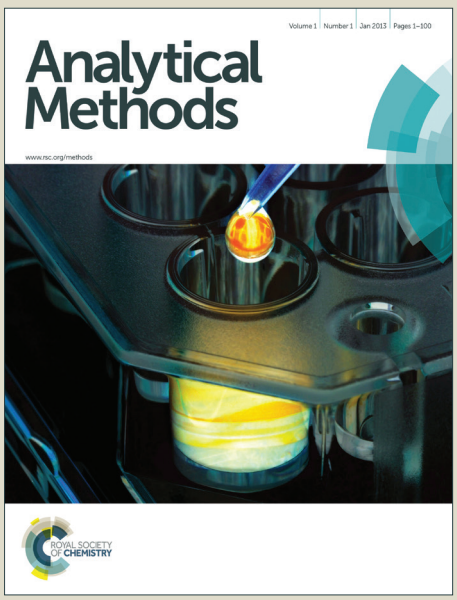

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PAPER

Quick determination of melamine in infant powder and liquid milk by Fourier Transform Infrared spectroscopy

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A simple, cost effective and environmental friendly analytical method was developed for quantification of Melamine (MEL) in liquid milk and infant powder by using transmission Fourier Transform Infrared (FT-IR) spectroscopy. Standards and samples were analyzed in the form of KBr pellet for recording FTIR spectra. Partial least square (PLS) calibration was established in the FT-IR region 851.62-798.39 cm^{-1} with linear range of 0.001-1%. The excellent coefficient of determination (R^2) 0.9999 was achieved with minimum errors in root mean square error of calibration (RMSEC) and root mean square error of prediction (RMSEP) with the value of 0.17 and 3.49 respectively. The accuracy of calibration model was also verified through root mean square error of cross validation (RMSECV) which was found to be 2.25. The LOD and LOQ of the method was 0.0001% (1 ppm) and 0.00035% (3.5 ppm) respectively. The method was successfully applied to liquid and infant powder milk samples with good recoveries (98.9-100.2%). The developed FTIR method is efficient, accurate and appropriate for MEL detection in the dairy industry with reduced cost and short analysis time.

1 Introduction

Melamine (2, 4, 6-triamino-1, 3, 5-triazine, C3H6N6) is an organic base chemical most commonly found in the form of white crystals of a trimer of cyanamide. MEL is produced in the large amounts primarily used as an industrial chemical in the synthesis of melamine formaldehyde resins for the manufacture of laminates, plastics, coatings, commercial filters, glues or adhesives and kitchenware[1, 2]. MEL has been illegally used for the intentional adulteration in milk and food products due to its high nitrogen content (66% by mass) which is often used as an indicator of protein content based on the Kjeldahl and/or Dumas analytical methods. These currently used methods for protein analysis cannot distinguish between nitrogen from protein sources and nitrogen from non-protein sources[3, 4].

According to the U.S. Food and Drug Administration (FDA) MEL and cyanuric acid concentrate, interact in the urine filled renal microtubules when they are absorbed into the bloodstream. In bloodstream MEL crystallize and form various round yellow colored crystals depending upon urine pH, these crystals block and damage the renal cells causing the kidneys to malfunction which can even lead to death in humans and animals[5]. In 2007, several raccoon dogs and cats were died in USA due to kidney failure by the intake of MEL tainted food[6,7]. Since the spring 2008, an increasing number of occurrences of kidney stones in infants were observed in China. Investigations has revealed that infant formula containing high levels of MEL were the cause of the urinary tract stone epidemic [8]. Due to these crises a safety limit of MEL intake has been officially set at 2.5 ppm for adult food and at 1.0 ppm for infant formula by the US-FDA[9]. The Chinese government legally regulated maximum residue level of MEL (1mg kg^{-1}) in infant formula after the health problems for thousands of infants in China [10]. Hence, the increasing adulteration of milk with MEL has promoted analytical methods which requires rapid and effective validations and sample investigations internationally. Different types of analysis methods have been developed for MEL detection such as, High performance liquid chromatography (HPLC)[1], Capillary electrophoresis (CE)[11], Ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS)[12], Gas chromatography/mass spectrometry (GC/MS)[13], Gas chromatography/tandem mass spectrometry (GC-MS/MS)[14], Enzyme-linked immunosorbent assay (ELISA)[15], Nanoparticals[16], Chemiluminescence[17] and Molecular imprinted polymer (MIP)[18]. The analysis of milk adulterated with MEL requires protein precipitation, centrifugation and liquid-liquid/ solid phase extraction (SPE) ; because protein contents interferes with matrix and reduce the detection efficiency of above techniques [19, 20]. These processes are complicated, laborious, time consuming and requires large amounts of organic solvents. Therefore, there is considerable tendency for simple techniques without pretreatment of the sample to simplify the examination of MEL and reduce the analysis time. In this respect transmission FT-IR spectroscopy has ability to analyze samples with high sensitivity, specificity and capability to serve as ''fingerprint'' technique with the application of chemometric analysis[21]. Raman spectroscopy is also used for the analysis of MEL because of its high-sensitivity less sample quantity but, it is difficult to analyze trace amount of the analyte due to weak scattering effect. This problem is solved by the development of surface-enhanced Raman scattering (SERS) spectroscopy and commonly SERS substrates used are; metal colloid, particularly silver colloid and nanoparticles[22-24]. However, with the transmission FT-IR spectroscopy the spectra could be recorded without any appreciable surface enhanced material. We have already developed the method using SB-ATR spectroscopy for the determination of MEL in dairy milk with better limit of detection (LOD) 2.5 mg kg^{-1} [25]. In this article, we have two objects: (i) to establish quick and sensitive analytical method with improved LOD compared with previous research work (ii) to develop a fast method using transmission spectroscopy with chemometrics for the assessment of MEL in liquid and infant powder milk samples. This approach provides a rapid and unique way of analysing real sample in its original form with no sample preparation.

2 Materials and Methods

2.1 Standard and samples

Melamine (99%) analytical grade was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Spectroscopic grade KBr was purchased from Merck (E. Merck, Darmstadt). For the dilution of standards raw milk was used as stock from dairy farm. The milk samples (infant powder/liquid) were collected from local market Hyderabad Sindh Pakistan.

2.2 Procedure for sample preparation

The liquid milk samples were freeze dried through Labcon freeze dryer (Labcon corporation Kansas City, Missouri, USA). The safest temperature was -4 to -40 ^{o}C used for drying milk sample. In this method except grinding no prior sample treatment is required, all milk samples were accurately weighed and grinded to fine powder in mortar and pestle to decrease the particle size. The KBr pellets were prepared by mixing 1mg milk samples with 99 mg of dried KBr, finely powdered and then thoroughly mixed in order to homogenize the mixture. The pellet was then condensed in the 13mm die at a pressure of 6 tons for 1 min and they were scanned in mid IR region on FT-IR spectrometer.

2.3 Preparation of standard

A stock powder of MEL and the freeze dried milk was prepared by intermingles of a 1:1 w/w ratio. The further dilution were proceeded by geometric mixing of equal mass ratios of freeze dried milk blend to 1%, 0.75%, 0.5%, 0.25%, 0.1%, 0.075%, 0.05%, 0.025%, 0.01%, 0.0075%, 0.005%, 0.0025%, 0.0015% and 0.001% MEL. The samples were prepared in triplicate and prior to analysis that were stored in sealed glass vials.

2.4 Optimization of FT-IR Spectral Features

Infrared spectra of standards and samples in the form of pellet were acquired with Thermo Nicolet 5700 FT-IR spectrometer equipped with removable KBr optics and deuterated triglycine sulphate (DTGS) detector. The FT-IR data investigation was conducted using infrared spectra analysis software package OMNIC (Thermo Nicolet Analytical Instruments, Madison, WI) in triplicate to obtain reproducible results. The recorded spectra were average of 32 scans per spectrum in mid Infrared region i.e. 4000-400cm^{-1} at 4cm^{-1} by optimizing resolution of the instrument. The fresh background spectrum from KBr pellet was recorded before obtaining spectrum of all pellets under the same instrumental conditions.

2.5 FT-IR Calibrations and statistical validation

A series of 14 calibration standards covering the range of 0.001-1% MEL were mixed in KBr to make each time the pellet of 100 mg by total weight to ensure homogeneity and required ratio. Turbo Quant (TQ) analyst software 7.2 programs (Thermo Electron Corporation, Madison, WI, USA, 2004) was applied to develop partial least square (PLS) calibration model based on standard spectra. The FT-IR spectra of standards were opened in TQ analyst program which is better option to save the time and labour to measure peak height, peak width and peak area as compare to manual calculations. The leave-one-out cross-validation procedure was used to verify the calibration model. The values of root mean square error of calibration (RMSEC) and coefficient of determination (R^2) were used as the validity criteria for calibration model. The predictive ability of PLS calibration model was further used to calculate the validation samples.

2.6 Validation of method

Validation of FT-IR was evaluated by standard addition of samples analyzed in triplicate. MEL free milk sample was prepared by spiking the known amount of MEL separately to make the concentrations of each sample at 10, 15, 25µg g^{-1} mL^{-1} for dried milk and infant powder milk.

Limit of detection (LOD) and limit of quantification (LOQ) of proposed method was measured by selecting the band area at low concentrations of standards, until the component related signal disappeared[26]. The LOD and LOQ were calculated by the following formulas:

$$LOD = 3 \times SD \times C/M \qquad LOQ = 10 \times SD \times C/M$$

Where: SD is the standard deviation; C is the concentration of analyte and M is the mean band area.

3 Results and discussion

FT-IR spectroscopy is a rapid analytical technique for both qualitative and quantitative analysis due to excellent performance of recently developed software's and chemometrics. PLS multivariate calibration model was applied in order to correlate the data collected between different variables on same observations in multi component mixtures. PLS works on principle of computing large amount of information which is common between different variables with maximal covariance[27]. A good calibration was generated with an excellent regression with the help of TQ analyst. Specific region of 851.62- 798.39 cm^{-1} for MEL was selected for best results. The invention of the calibrations was evaluated by running the samples of known melamine profile. To estimate the strength of the models to fit the calibration data and to calculate the deviation of the models, root mean square error of calibration (RMSEC), root mean square error of prediction (RMSEP) and root mean square error of cross validation (RMSECV) were used.

In Fig. 1, typical FT-IR transmittance spectra of MEL standard and pure milk are shown. The IR transmission peaks at 3500-3000 and 1700-1300 cm^{-1} in the MEL spectrum are attributed to the stretching and bending vibrations of amino groups present in the MEL. The characteristic band of MEL standard was observed in transmission spectrum, which is explained in Table 1. The absorption band at 814.0 cm^{-1} is characteristic of out-of plane bending of 1,3,5-s-triazine ring of MEL which was absent in pure milk spectrum, therefore the region 851.62- 798.39 cm^{-1} was selected for calibration.

Table 1 Characteristic bands of Melamine standard observed in FTIR transmission spectrum.

Observed wavenumber cm^{-1}	Assignments
3469.0–3419.0	NH2 stretch, typical of MEL, absent in milk.
3333.7	Asymmetric NH2 stretch
3132.0	Symmetric NH2 stretch
1653.7	NH2 deformation
1559.2	Quadrant stretching of 1,3,5-s-triazine ring
1438.2	Semicircle stretching of 1,3,5-s-triazine ring
1027.5	C–N stretching of primary amines, tertiary C
814.0	Out of plane ring bending by sextants of 1,3,5-s-triazine

As depicted in Fig. 2, the spectra of 14 standards prepared with different concentration of MEL shows the good linearity of the calibration model in the selected region. The selection of FT-IR spectral statistical treatment was based on the highest values of R^2 and the lowest values of RMSEC obtained during developing PLS model. Firstly the PLS model was tested with different factors which effects on the R^2 and RMSEC of the method through validation point. The method was also checked with two point baseline (852.38-798.39 cm^{-1}) calibration which shows the good correlation coefficient (R^2 = 0.999) exists between actual and predicted values. Diminutive values of RMSEP (3.49) and RMSEC (0.17) with higher number of factor indicates precision of the method as shown in Table 2.

Table 2 PLS parameters tested with validation points.

With validation points	Factor	R^2	RMSEC	RMSEP
Baseline (None)	4	0.995	3.27	7.42
	5	0.998	2.19	5.00
	6	0.999	1.66	3.65
	7	0.999	0.738	2.49
Two point baseline	4	0.998	2.17	4.00
(852.38-798.39)	5	0.999	1.54	3.34
	6	0.999	0.927	2.43
	7	0.999	0.173	2.17

Cross validation with the leave out one standard point with similar previous parameters was carried out in order to verify that the model will perform well when applied to new samples. Table 3 illustrate the RMSECV and R^2 values obtained with cross validation points; best results were obtained with two point baseline and 7 factors (R^2 = 0.994, RMSECV = 2.25). Fig. 3 shows the plot of RMSECV as the function of the variables. After the first factor the RMSECV decreases rapidly until it reached at plateau with the maximum number of PLS factors before the decrease in the RMSECV is negligible.

Table 3 PLS parameters tested with cross validation point.

Cross validation	Factor	R^2	RMSECV
Baseline (None)	4	0.987	5.69
	5	0.986	5.54
	6	0.995	3.52
	7	0.993	2.48
Two point baseline	4	0.982	6.42
(852.38-798.39)	5	0.986	6.24
	6	0.993	4.10
	7	0.994	2.25

Before the application to the detection of real samples the statistical parameters were validated as described above. Three different spiked concentrations (10, 15 and 25 µg g^{-1} or mL^{-1} with 3 similar measurements) of liquid milk and infant powder were implemented to examine the recovery and precision of the method as mention in Table 4. The relative recoveries ranged 98.9-100.2% with relative standard deviations (RSD) from 1.38-2.07%. A standard at the levels that produced distinguishable signal from the baseline noise was used to determine the LOD based on signal to noise ratio of 3. The LOD and LOQ were 0.00015% and 0.00035% corresponding to 1 and 3.5 mg kg^{-1} respectively.

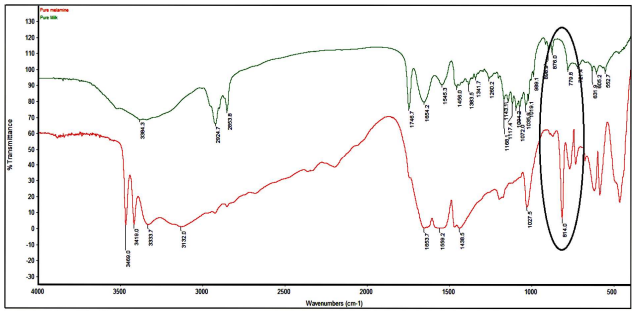

Figure 1 Transmission FT-IR spectra of pure MEL and pure milk.

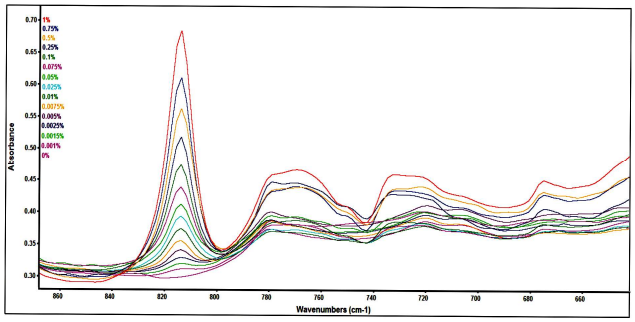

Figure 2 The spectra of 14 standards calibration prepared with different concentration of MEL in milk on transmission FT-IR spectroscopy.

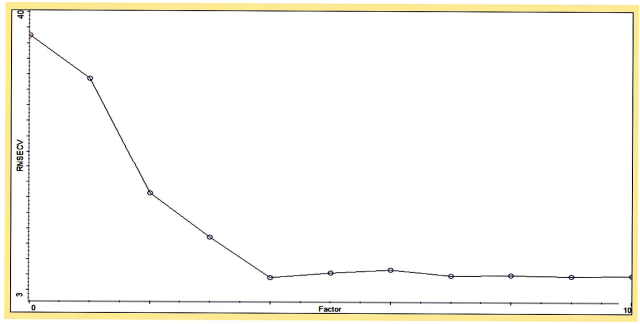

Figure 3 The plot of RMSECV with the leave out one standard point for the optimization of MEL content PLS calibration.

The comparison of the proposed method with earlier reported work in term of total detection time, linearity and LOD is presented in Table 5. The LODs of HPLC-UV, HILIC-UV and GC-MS/MS method is reported as 0.2, 0.003 and 0.01mg kg^{-1} respectively. The HPLC method has low limit of detection as compared with our method but required higher analysis time. Moreover HPLC-UV methods are not able to confirm the target analyte, because the UV spectra of MEL exhibits absorption bands below 250 nm, erroneous quantification can occur if insufficient care is paid to chromatographic conditions or sample preparation[28]. GC-MS methods offers better detection limit i.e. 0.01 mg kg^{-1}; however derivatization is required with extensive sample pretreatment including extraction and purification which are costly and time consuming[29]. Tedious sample preparation and clean up make the methodology impractical and inefficient for analyzing a large number of samples, so a simple, effective, reliable method for detecting MEL and its analogs in food is needed. The present method does not require any sample pretreatment and derivatization. It's a simple, cost effective

method because the FT-IR instruments are available in many countries. The MIP and ELISA methods have good linearity with

Table 4 Result of the determination of melamine in raw and powder milk.

Milk sample	Original amount	Added (μg mL^{-1} or g^{-1})*	Found (μg mL^{-1} or g^{-1})	Recovery (%)	RSD (% n=3)
Infant powder milk	-	10	9.92	99.20	1.90
	-	15	14.97	99.80	1.49
	-	25	25.05	100.20	1.38
Liquid milk	-	10	9.89	98.90	2.07
	-	15	14.93	99.54	1.70
	-	25	25.03	100.12	1.51

* μg mL^{-1} for raw milk and μg g^{-1} for powder milk sample

Table 5 Previously published analytical methods in term of LOD and total detection time for determination of melamine in milk and dairy products.

Methods	Matrixes	Linear range	Total time to detect	Detection limit	Ref:
HPLC-UV	Infant Formula	5-40 mg mL^{-1}	1+ h[a]	0.2μg mL^{-1}	[1]
CZE-UV	Milk, milk powder, fish feed	0.8-80μg mL^{-1}	2+ h[a]	0.08μg mL^{-1}	[11]
UPLC-MS/MS	Milk, bakery goods, flour	0.5-40 mg kg^{-1}	>1+ h[a]	0.2mg kg^{-1}	[12]
MIP	Milk sample	0.005–1μmol L^{-1}	1+ h[a]	0.07nmol L^{-1}	[30]
ELISA	Liquid, powder milk, dog food	0.03-9ng mL^{-1}	2+ h[a]	0.01ng mL^{-1}	[31]
GC-MS/MS	Dairy product	0.05-2 mg kg^{-1}	1+ h[a]	0.01mg kg^{-1}	[29]
ATR-FTIR	Milk liquid	25-0.0625 %	2 min	0.00025%	[25]
HILIC-UV	Dairy product	0.005-32mg L^{-1}	2+ h[a]	0.003mg L^{-1}	[32]
FTIR-KBr	Liquid milk, powder milk	0.001-1%	4 min	0.0001%	Current Study

lower limit of detection and sensitivity for MEL residue determination in various matrices. The disadvantages are that the analysis is only semi quantitative and it sometimes gives rise to false positives. ELISA requires a specific antibody against the analyte, which requires considerable work[33]. Previously we have reported SB-ATR spectroscopy for the MEL detection[25], but present study offers better detection limit (2.5 vs. 1 mg kg^{-1}) and accuracy using Transmission mode of FT-IR. ATR mode has two main difficulties; one is the less sensitivity owing to the short path length of ATR cell (~2-4 cm^{-1}) in comparison to transmission mode and other is development of accurate calibration curve on ATR crystal, especially in the case of present study for the solid standards of various concentrations. Therefore, transmission FT-IR is the excellent choice for the accurate determination of MEL in infant powder and liquid milk without using any solvent and pretreatment of the sample.

4 Conclusion

The proposed FT-IR method achieved analytical simplicity, better accuracy and improved sensitivity for quantification of MEL. It eliminates the intricacy of usual extraction methods allowing quick and green methodology without using hazardous organic chemicals. The multivariate calibration technique (PLS) enables the determination of MEL in liquid and infant powder milk with good accuracy and precision. LOD and LOQ of the method was found to be 1 ppm and 3.5 ppm with excellent percent recoveries (98.90-100.12); the proposed method could be efficiently applied for routine determination and quantification of MEL in adulterated powder and liquid milk with quite satisfactory precision and accuracy.

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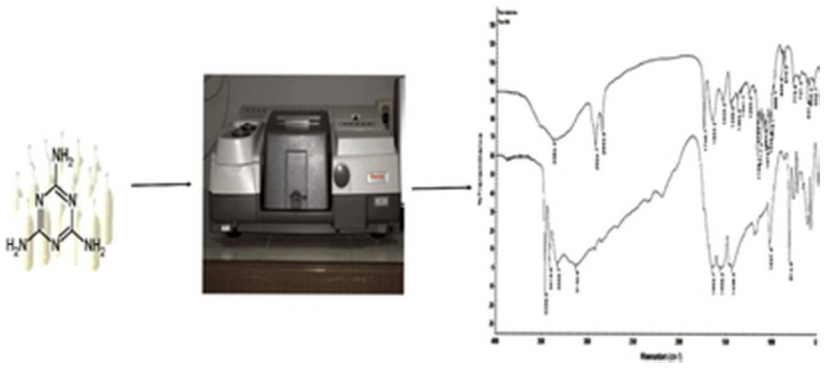

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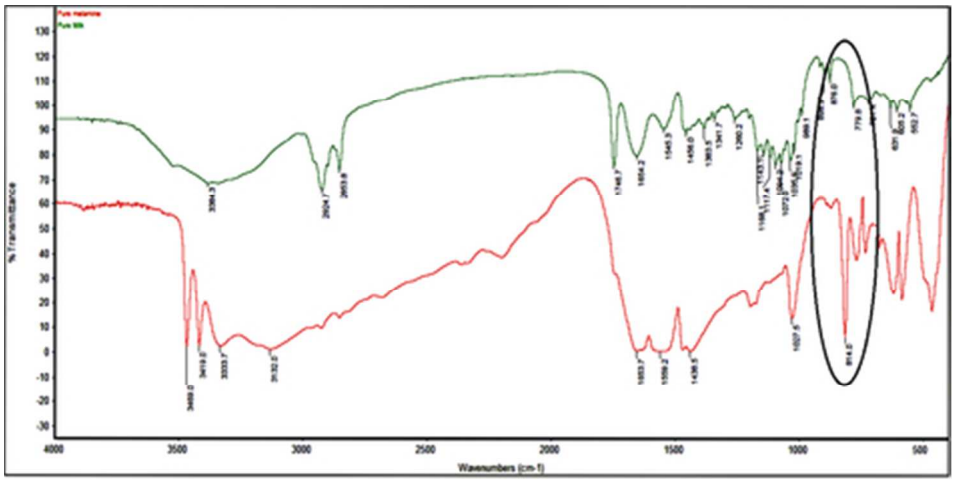

Figure 1 Transmission FT-IR spectra of pure MEL and pure milk
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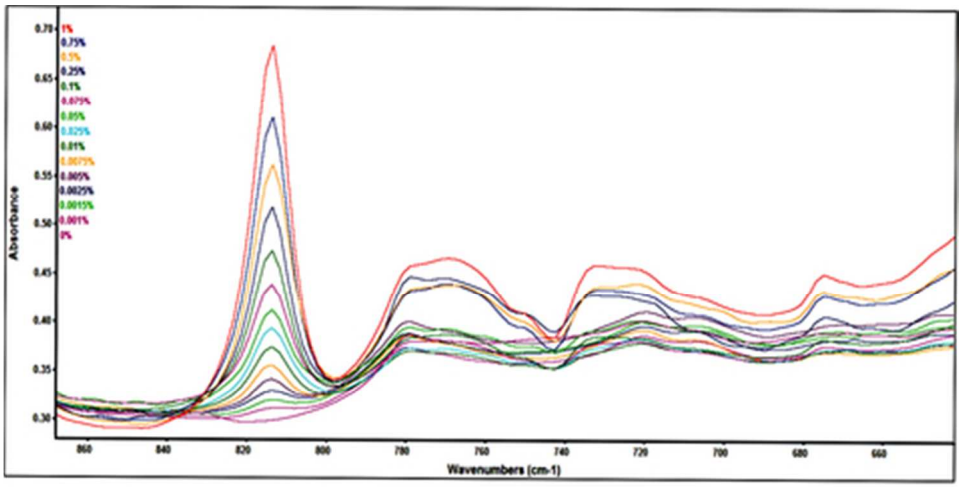

Figure 2 The spectra of 14 standards calibration prepared with different concentration of MEL in milk on transmission FT-IR spectroscopy
40x20mm (300 x 300 DPI)

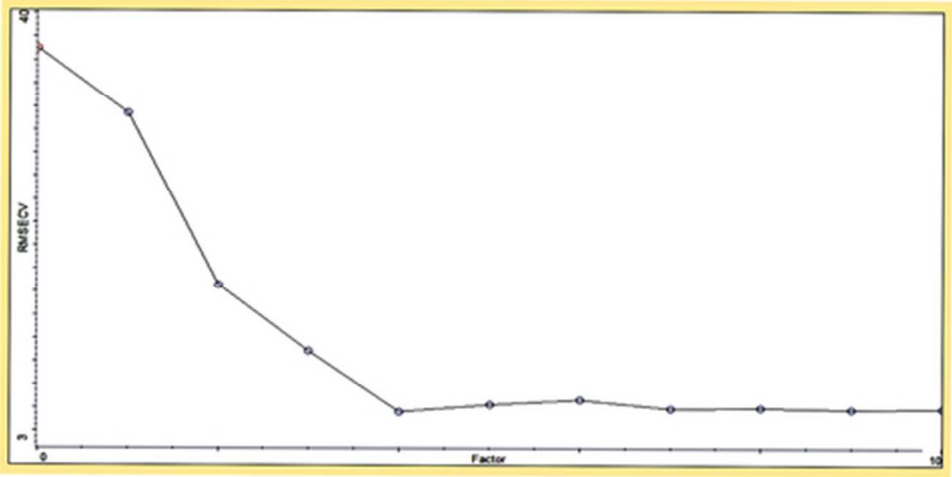

Figure 3 The plot of RMSECV with the leave out one standard point for the optimization of MEL content PLS calibration
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