Printed in the Republic of Korea
ANALYTICAL SCIENCE & TECHNOLOGY
Vol. 28 No. 1, 1-7, 2015
http://dx.doi.org/10.5806/AST.2015.28.1.1

Development of a method for the determination of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in dust using liquld chromatography tandem mass spectrometry

W. K. Lee, S. J. Kang[1], J. E. Oh[1], S. H. Hwang[1] and D. H. Lee[1,★]

Department of Laboratory Medicine, National Cancer Center
[1]Department of Laboratory Medicine, National Cancer Center, 323 Ilsan-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do 410-769, Korea
(Received January 16, 2015; Revised February 3, 2015; Accepted February 6, 2015)

LC-MS/MS를 이용하여 먼지 속의 NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) 정량 분석법 개발

이원경 · 강수진[1] · 오지은[1] · 황상현[1] · 이도훈[1,★]

국립암센터 진단검사의학과, [1]국립암센터 진단검사의학과
(2015. 1. 16. 접수, 2015. 2. 3. 수정, 2015. 2. 6. 승인)

Abstract: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco specific nitrosamine found only in tobacco products. The ability to monitor biomarker concentrations is very important in understanding environmental tobacco smoke (ETS). In this study, an efficient and sensitive method for the analysis of NNK in dust was developed and validated using liquid chromatography tandem mass spectrometry. Dust was collected with filter paper soaked in methanol. The standard solution and dust sample were diluted with 100 mM ammonium acetate and extracted using dichloromethane. Our calibration curves ranged from 25 to 10^4 pg/mL. Excellent linearity was obtained with correlation coefficient values between 0.9996 and 1.0000. The limit of detection (LOD) was 5 pg/mL (S/N ≥ 3) and the retention time was 10 min. The limit of quantification (LOQ) was 25 pg/mL, and the acceptance criteria was the rate of 98-103% (80-120% at levels up to 3×LOQ). The coefficient of variations (CV) was 2.8%. Accuracies determined from dust samples spiked with four different levels of NNK racurves ranged that from 25 to 104 pg/mL. Excellent linearity was obtained between 92.1% and 114%. The precision of the method was acceptable (5% of CV). The recovery rates of the whole analytical procedure at low, medium, and high levels were 105.7-116.5% for NNK. The carry-over effects during LC-MS/MS analysis were not observed for NNK. This manuscript summarizes the scientific evidence on the use of markers to measure ETS.

Key words: 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) Environmental tobacco smoke (ETS) Tobacco-specific nitrosamines (TSNAs) LC-MS/MS Dust

★ Corresponding author
Phone : +82-(0)31-920-1734 Fax : +82-(0)31-920-1339
E-mail : dhlee@ncc.re.kr

1. Introduction

The fact that people are aware of the dangers of smoking and second-hand smoke (SHS) has been found in many studies already. Liver cancer is one of the common cancers all over the world. The International Agency for Research on Cancer (IARC) found smoking is one of the risk factors for liver cancer. Also many studies have demonstrated that SHS is a main cause of cardiovascular systems and lung cancer.[1-2] SHS includes many chemical compounds and associated nitrosamines as well as other carcinogenic impurities, polycyclic aromatic hydrocarbons (PAHs), 3-ethenylpyridine, phenol, cresols, naphthalene, and formaldehyde.[3-6] Tobacco-specific nitrosamines (TSNAs) are the strongest carcinogens in tobacco and tobacco smoke. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) TSNAs are found only in tobacco products. In humans, NNK is almost changed to 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). TSNAs are classified as lung carcinogens by the IARC.[1,7-9,22] If it is exposed to such chemical components in home and schools, restaurants, bars, etc. This is referred to environmental tobacco smoke (ETS). Recently, smoke free of non-smoking legislation was enforced in public places such as restaurants, bars and work places. However, the problem still exists at homes where children are dangerously exposed to ETS.[10] Another study indicated that when the father smokes in the home, then preschool children are more affected by SHS than other nonsmoking household members. Also a study demonstrateds that outdoor-smoking may reduce exposure to SHS, but it does not protect the family completely.[11] Presented at various research, exposure of ETS has very harmful effects on newborns and children. It is particularly dangerous due to abnormalities in lung function and behavioral problems in babies.[12-17] Smoking by men in the family has a serious impact on women and fetuses. Children are exposed to ETS not only in their homes but also in schools, restaurants, childcare facilities and other public places.[18] This problem is not confined to children. People do not know that how much they are exposed to SHS in everyday life. However exposure to secondhand smoke involves not many ingredients that can be quantified. NNK is a good indicator of how to quanitify SHS. Existing studies related to NNK have been collected from homes, using vacuum cleaners.[19-20] These methods are inconvenient for mobility and collection characteristics. Therefore in this study, we utilized a simple and rapid method for the detection and quantification of NNK in dust.

2. Experimental Method

2.1. Materials

All solvents were high performance liquid chromatography (HPLC)-grade. Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol was purchased from Merke (Darmstadt, Germany). Water and dechloromethane used were of analytical grade (Fisher Scientific UK Limited). NNK and NNK-d4 were purchased from Toronto Research Chemical (North York, Ontario, Canada).

2.2. Collection and Extraction

Dust was collected with filter paper soaked in 50% methanol and added to 4 ml of 100 mM ammonium acetate after drying. The filter paper was removed after being voltexed for 15 min. The samples were mixed with internal standards 50 μL of NNK-d_3 (10000 pg/mL), and extracted with 5 mL of dichloromethane. Next, extracts were evaporated for 20 min at 50 °C and redissolved in 135 μL of solution with 10% methanol containing 10 mM ammonium acetate. Next, the solution had the pH adjusted to 6.5 for LC-MS/MS analysis. before it (10 μL) was into the LC-MS/MS system.

2.3. Instrument and analytical conditions

All samples were analyzed using Agilent 1260 Infinity rapid resolution liquid chromatograph from Agilent Technologies (Santa Clara, CA, USA) coupled with a Triple Quad 5500 equipped with a TurboIonSprayTM source from AB SCIEX (Framingham, MA, USA). A poroshell 120 EC-C18 column 4.6×50 μm, 2.7 μm from Agilent Technologies (Santa Clara,

CA, USA) was used for LC seperation. LC conditions were as follows: column temperature, 40 °C; mobile phase, solvent A (10 mM ammonium acetate in water) and solvent B (10 mM ammonium acetate in methanol); flow-rate, 0.6 mL/min; the inject volume, 5 μL. A linear gradient condition was used as follows (time, solvent A:solvent B):0-1 min, 80:20-30:70; 2-7 min, 0:100; 8-10 min, 80:20. ESI-MS conditions were as follows: nebulizer gas, N2 (50 psi); ionspray voltage, 5000 V; the turbo ion spray temperature, 500 °C; declustering potential, 60 V; entrance petential, 10 V; collision energy, 30 V; the dwell time, 300 ms; ionization mode, positive ion. NNK and NNK-d_4 were assayed by quantifying the multiple reaction monitoring (MRM) transition of $[M+H]^+$ ion of NNK at m/z 208→122 and NNK-d_4 at m/z 212→126.

2.4. Calibration

The method was calibrated by spiking a pooled blank dust solution with 25, 50, 125, 250, 1000, 2500, 5000, 7500 and 10000 pg/mL NNK. Method validation was performed with an injection volume of 10 μL. The background peak area ratio in unspiked dust solution was zero for NNK. Each calibrator was analyzed twice. The means of the analyte/internal standard ratio were used for calculation of the regression function.

2.5. Method validation

As a criterion, the accuracy at average concentration level tested in these six matrices should be in the range of 85-115%. The recovery rate was determined by comparing the analyte concentrations at the low, middle and high levels, that were measured when blank dust extracts were spiked at the beginning of the sample work-up procedure, after sample work-up, and before the LC-MS/MS measurements. Acceptance criteria for precision were ±15% to ±20% at levels up to three times the LOQ. Accuracy at low, medium, and high concentrations were determined with pooled blank dust solutions spiked at low, medium and high levels. Each level was tested five times. Acceptance criteria were rates of 100±15% (LLOQ:100 ±20%), as well as precision ≤ 15% (LLOQ:≤ 20%). LOD and LOQ were estimated with the signal to noise ratios of ≥ 3 and ≥ 5 were applied for estimating the LOD and LOQ, respectively. Matrix effects were determined by calculating the peak area ratios of the analytes at low and high concentrations when spiked to work-up dust extracts and the same amounts of analytes in six different blank matrices (Matrix factor-CV ≤ 15%). The carry over effects on the chromatographic system were tested by injecting dust extracts with low and high analyte concentrations two times, followed by a blank injection. These experiments were repeated three times (Blank ≤ 20% LLOQ). The freeze-thaw stability was confirmed subsequently at room temperature for 24h, refrigeration temperature (-20 °C) and freezing temperature (-80 °C). The method was validated according to the U.S. Food and Drug Administration (FDA)/CDER guidelines for bioanalytical methods.[21]

2.6. Data analysis

Calibration curves were prepared using a linear regression with 1/x weighting. All standard and sample concentrations were determined using internal standard areas versus analyte areas. Data analysis was performed using Microsoft Office Excel 2007.

3. Results and Discussion

3.1. LOD and LOQ

The LOD and LOQ for dust NNK method were assessed using NNK in methanol samples of the lowest concentration for the standard curve. The LOQ sample was processed and analyzed with a calibration curve and QC sample. The LOD and LOQ were 5 pg/mL (S/N 3.2), 25 pg/mL (S/N 7.2), respectively. The LOD and LOQ were defined by peak height, higher than the maximum baseline height of blank with the signal to noise ratios of ≥ 3, and ≥ 5 were applied for estimating the LOD and LOQ, respectively. The mean accuracy and % CV (precision) for the LOQ were 99.9% and 2.7% respectively. A typical MRM chromatogram at the

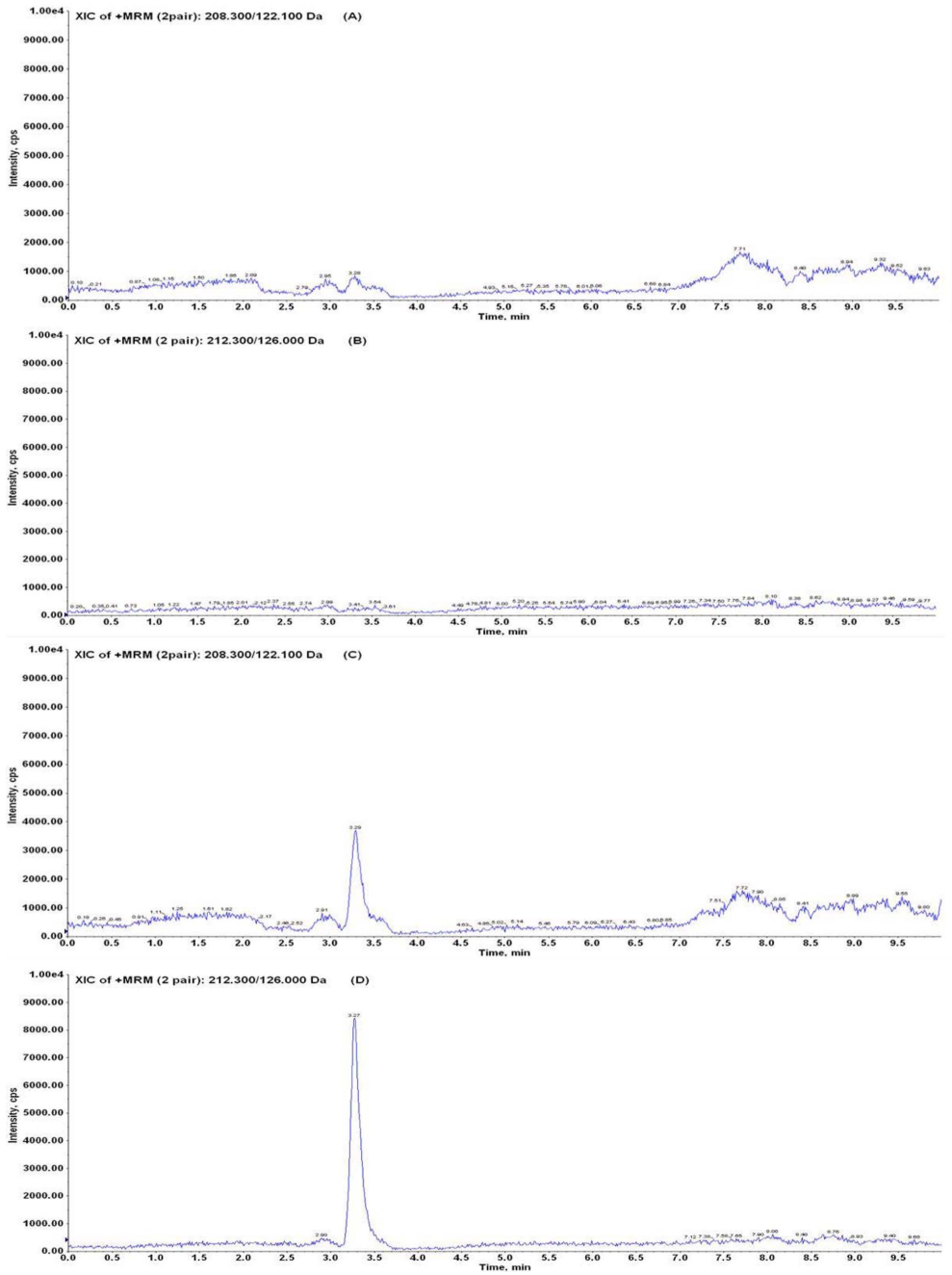

Fig. 1. Representation of MRM chromatograms of NNK and IS in blank dust. [A]Blank dust [B]IS of blank, [C]NNK spiking in blank dust (at the LOQ concentration) [D]IS (at 10000 pg/mL of NNK-d3)

LOQ concentration is shown in *Fig.* 1.

3.2. Calibration, accuracy and precision

The accuracy and precision of the dust NNK method were assessed by analyzing QC samples with a calibration curve of three different days. The calibration curve consisted of seven standards of different concentrations, each in duplication. Our

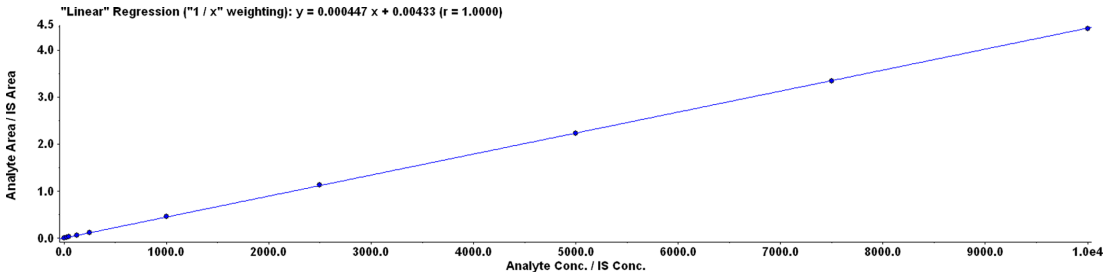

Fig. 2. Calibration curve for validation range from 25 to 10000 pg/mL.

Table 1. Accuracy and precision obtained for QC samples from blank dust

Conc (pg)	Within day (n=8)						Between day (n=24)					
	Precision			Accuracy			Precision			Accuracy		
	Mean	SD[a)]	CV%	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%
30	30.4	1.4	4.7	101.3	4.8	4.8	31.2	0.5	1.6	104.4	1.8	2.0
450	475.2	11.2	2.4	105.8	2.6	2.4	467.2	11.0	2.4	104.6	2.4	1.5
4500	4523.3	182.4	4.0	100.5	4.0	4.0	4508.3	152.8	3.4	100.6	3.2	0.4
9000	9050.0	125.8	1.4	100.7	1.5	1.5	9051.7	347.4	3.8	100.6	3.9	0.2

[a)]SD-standard deviation

calibration curves ranged from 25 to 10000 pg/mL. Excellent linearity was obtained with correlation coefficient values between 0.9996 and 1.000, and the corresponding equation was y = 0.0102x+0.399 (as shown in *Fig.* 2.) The QC sample consisted of four concentrations. The accuracies determined in four different dust matrices were between 100.5 and 104.6%. Precision (both within- and between days) of the method was found to be acceptable (CV < 10%). as shown in *Table* 1.

3.3. Selectivity and specificity

Six different dust samples were used to check the selectivity and specificity of the dust NNK method. Also no interfering peaks from the different dust samples were found. Similarly, six different samples of dust were analyzed with and without internal standard to ascertain the selectivity and specificity of the dust NNK method. As shown in *Table* 2, significant interfering peaks from the six different dust samples were not found at the retention times and in the MRM channels of either the analytes of the internal standards.

Table 2. Selectivity and specificity of six different dust samples

No	Blank	Blank + Spiked NNK[a)]	% bias
1	3950	20300	19.5
2	3880	20200	19.2
3	1470	23100	6.4
4	1660	21500	7.7
5	4390	22600	19.4
6	3420	23900	14.3
Mean	3128	21933	14.4

[a)]Spiked amount : 25 pg/mL

3.4. Recovery

The extraction recovery of NNK rates of the whole analytical procedure was at 30, 450, 4500 and 9000 pg/mL. The extraction recoveries of assay were 105.7, 111.3, 108.1 and 116.5%, respectively. The

Table 3. The extraction recovery of NNK

Concentration (pg)	%Total Recvery	%Extraction deficiency	%Matrix effect
30	105.7	117.2	90.2
450	111.3	143.0	85.0
4500	108.1	140.6	85.0
9000	116.5	153.5	86.9

Table 4. Stability parameters of NNK under different conditions (n=8)

Conc (pg)	Precision (%)				Accuracy (%)			
	Daily QC	Freeze	Refrigeration	Room temp	Daily QC	Freeze	Refrigeration	Room temp
30	30	32	33	30	102	108	112	102
450	475	480	484	447	106	107	108	99
4500	4450	4290	4570	4190	98	95	101	93
9000	8830	9090	8980	8310	98	101	99	92

Table 5. Carry-over between samples of blank and LLOQ, HLOQ of NNK

No	Analyte %	IS %
1	12.7	0.0
2	16.2	0.1
3	9.0	0.0
Mean	12.6	0.0

results of the investigated recovery parameters are shown in *Table* 3.

3.5. Stability

The results of the investigated stability parameters are shown in *Table* 4. The stability of NNK was studied at -20 ºC, room temperature, -80 ºC and after freeze-thaw cycle. For the dust NNK method, QC samples at 30, 450, 4500 and 9000 pg/mL were used for the stability study. Thus, NNK was found to be stable for at least at room temperature 23h, for at least 4 weeks at -20 and -80 ºC. NNK was found to be stable for at least three freeze-thaw cycles.

3.6. Carry-over and matrix effect

The carry over in the blank sample following the high concentration standard was smaller than 15% of the LOQ and 1% for the internal standard. as shown in *Table* 5. Consequently, the carry-over effect between samples was not observed. The matrix effect on LC/MS/MS was analyzed using six different dust samples of the low and high QC samples. The matrix effect on the ionization of the analyte and intetnal standard did not interfere with the assay conditions.

4. Conclusions

We do not know that how much of our lives are exposed to SHS. People know and acknowledge the dangers of SHS. However, they do not recognize the risks of being exposed to SHS in places such as buildings, institutions, restaurants, etc. Therefore, we think that people have not made a strong demand to a nation about smoking restrictions. NNK is an indicator of smoking and SHS. This study is the first time that a simple and rapid method for the detection and quantification of NNK in dust was explored. The excellent sensitivity of the proposed method makes it applicable for the determination of NNK studies on exposure to ETS. For previous studies, NNK was collected using a vacuum cleaner. However, this method was inefficient and was not simple. The main point of this very simple method herein is to demonstrate how easy it is to be exposed to ETS wherever we live. If so, the general public would know quantitatively how much they are exposed to ETS in their living environment. It is expected to reduce ETS exposure and change the awareness of society about its health.

Acknowledgements

This work was supported by research grants from the National Cancer Center (1310060), Korea.

References

1. Lyon, In ‘International Agency for Research on Cancer’, Monogr Eval Carcinog Risks Hum, 83, p1-1438, France 2004.
2. W. O. Spitzer, V. Lawrence, R. Dales, G. Hill, M. C. Archer, P. Clark, L. Abenhaim, J. Hardy, J. Sampalis and S. P. Pinfold, *Clin Invest Med.,* **13**(1), 17-42 (1990).
3. R. A. Jenkins, M. R. Guerin and B. A. Tomkins, ‘The

Chemistry of Environmental Tobacco Smoke: Composition and Measurement'. Lewis Publisher, Boca Raton, Florida, 2000.
4. D. Hoffmann, I. Hoffmann and K. El Bayoumy, *Chem. Res. Toxico.*, **14**, 767-790 (2001).
5. G. E. Matt, P. J. Quintana, H. Destaillats, L. A. Gundel, M. Sleiman, B. C. Singer, P. Jacob III, N. Benowitz, J. P. Winickoff, V. Rehan, P. Talbot, S. Schick, J. Samet, Y. Wang, B. Hang, M. MartinsGreen, J. F. Pankow and M. F. Hovell, *Environ Health Perspect*, **119**, 1218-26 (2011).
6. Lyon, In 'International Agency for Research on Cancer', Monogr Eval Carcinog Risks Hum, 37, p1-291, France, 1985.
7. J. M. Yuan, W. P. Koh, S. E. Murphy, Y. H. Fan, R. W. Wang, S. G. Carmella, S. M. Han, K. Wickham, Y. T. Gao, M. C. Yu and S. S. Hecht, *Cancer Res.*, **69**, 2990-2995 (2009).
8. S. S. Hecht, *Chem. Res. Toxicol*., **11**, 559-603 (1998).
9. A. Burton, *Environ Health Perspect*, **119**, A70-4 (2011).
10. J. P. Winickoff, J. Friebely, S. E. Tanski, C. Sherroda, G. E. Matt, M. F. Hovell and R. C. McMillen, *Pediatrics*, **123**, e74-9 (2009).
11. M. W. Seong, J. S. Moon, J. H. Hwang, H. J. Ryu, S. J. Kang, S. Y. Kong, T. H. Um, J. G. Park, J. S. Lee and D. H. Lee, *Clinica Chimica Acta*, **411**(1-2), 72-76 (2010).
12. L. Gatzke-Kopp and T. Beauchaine, *Child Psychiatry Hum Dev*, **38**, 255-69 (2007).
13. C.-J. Hsieh, S.-F., Jeng, Y.-N. Su, H.-F. Liao, W.-S. Hsieh, K.-Y. Wu and P.-C. Chen, *Nicotine Tob Res*., **12**, 1108-1117 (2010).
14. J. Makin, P. A. Fried and B. Watkinson, *Neurotoxicol Teratol*., **13**, 5-12 (1991).
15. D. P. Strachan and D. G. Cook, *Thorax*, **52**, 905-14 (1997).
16. D. G. Cook and D. P. Strachan, *Thorax*, **52**, 1081-94 (1997).
17. D. G. Cook, D. P. Strachan and I. M. Carey, *Thorax*, **53**, 884-93 (1998).
18. S. H. Hwang, J. H. Hwang, J. S. Moon and D. H. Lee, *Korean J Pediatr*, **55**(2), 35-41 (2012)
19. G. E. Matt, P. J. E. Quintana, M. F. Hovell, J. T. Bernert, S. Song, N. Novianti, T. Juarez, J. Floro, C. Gehrman, M. Garcia and S. Larson, *Tob Control*, **13**, 29-37 (2004).
20. N. Ramirez, M. Z. Ozel, A. C. Lewis, R. M. Marce, F. Borrull, J. F. Hamilton, *J. Chromatogr A*., **1219**, 180-187 (2012).
21. Food and Drug Administration, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatory Information/Guidances/UCM070107, Accessed 17.04.09.
22. H. Hongwei, Z. Xiaotao, T. Youngfeng, T. Gangling, L. Yulan, H. Qingyuan, Tian Youngfeng, Tang Gangling, Liu Yulan and Hu Qingyuan, *J. Pharm. Biomed. Anal*, **63**, 17-22 (2012).