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Rapid multi-class multi-residue method for the confirmation of chloramphenicol and eleven nitroimidazoles in milk and honey by liquid chromatography tandem mass spectrometry

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Abstract

A confirmatory method has been developed to allow for the analysis of eleven nitroimidazoles and also chloramphenicol in milk and honey samples. These compounds are classified as A6 compounds in annex IV of council regulation 2377/90 and therefore prohibited for the use in animal husbandry. Milk samples are extracted by acetonitrile with addition of NaCl, while honey samples are first dissolved in water before a similar extraction. Honey extracts undergo a hexane wash to remove impurities. Both milk and honey extracts are evaporated to dryness and reconstituted in initial mobile phase. These are then injected onto an LC-MS/MS system and analysed in less than 9 minutes. The MS/MS is operated in MRM mode with positive and negative electrospray ionisation. The method is validated in accordance with Commission Decision 2002/657/EC and is capable of analysing for metronidazole, dimetridazole, ronidazole, ipronidazole and there hydroxy metabolites MNZ-OH, HMMNI and IPZ-OH. The method is also able to analyse for carnidazole, ornidazole, ternidazole, tinidazole and chloramphenicol. A level of 3 µg L^{-1} / µg kg^{-1} (RL) for MNZ, DMZ and RNZ has been recommended by the community reference laboratory responsible for this substance group and this method can easily detect all nitroimidazoles at this level. A minimum required performance level of 0.3µg L^{-1} / µg kg^{-1} is in place for chloramphenicol which the method can also easily detect. For nitroimidazoles the decision limits (CCα) and detection capabilities (CCβ) range from 0.41 to 1.55µg L^{-1} and 0.70 to 2.64µg L^{-1} respectively in milk and from 0.38 to 1.16µg kg^{-1} and 0.66 to 1.98µg kg^{-1} respectively in honey. For chloramphenicol the values are 0.07 and 0.11µg L^{-1} in milk and 0.08 and 0.13µg kg^{-1} in honey. Validation criteria of accuracy, precision, repeatability and reproducibility along with measurement uncertainty are calculated for all analytes in both matrices.

Keywords: Nitroimidazoles; chloramphenicol; milk; honey; lc-ms/ms; validation.

1. Introduction

1.1. Nitroimidzoles

5-Nitroimidazoles are primarily used for the prophylactic and therapeutic treatments of diseases such as histominiasis and coocidiosis in poultry, hemorrhagic enteritis in pigs and genital tricchoniasis in cattle. Recently, reports from China suggest that the use of nitroimidazoles in beekeeping is being practiced [Zhou et al., 2007.]. Nitroimidazoles may be used to prevent and control Nosema apis in hives. Nosema Apis is a microsporidian pathogen that is commonly found in Apis mellifera throughout the beekeeping world. [Official Method (2003)]

Nitroimidazoles(NMZs) are believed to be carcinogenic and mutagenic to humans and as a consequence are placed in the group A6 (prohibited) substances and their use in food producing animals within the European Union is not permitted under Regulation 2377/90. They are also prohibited for use in the U.S.A and China. The analysis of these compounds is required under Council Directive 96/23/EC. There is a need for rapid multi residue analytical methods that have the capability to include a wide rage of these analytes in all matrices where abuse may be found. The structure of these 5-nitroimidazoles and their metabolites are seen in table 1.

The Community Reference Laboratory (CRL) in Berlin has carried out homogeneity and stability studies on NMZs in various matrices [Polzer et al., 2004, Polzer et al., 2005]. These studies showed that there is not a homogenous distribution of these analytes in turkey muscle and they also observed a rapid degradation in analyte concentration stored for prolonged periods above 4 °C. In contrast it was discovered that for plasma, retina and egg samples; the analytes were stable during storage under the same conditions which resulted in stable concentrations and allowed detection of these compounds for longer periods after medication had been halted. Therefore, it is advised that plasma, retina and eggs be used as target matrices for the

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residue control of NMZs [Polzer et al., 2004, Polzer et al., 2005].

As a result of these published studies there has been an increase in the methods being developed for the analysis of these compounds in matrices such as egg [Mottier et al. 2006, Mohammed et al., 2008, Xia et al., 2006, Daeseleire et al., 2000, Cronly et al., 2009(a)] and plasma [Aerts et al., 1991, Fraselle et al., 2007, Thompson et al., 2009, Cronly et al., 2009(b)]. The majority of methods published for the analysis of eggs involve extraction with acetonitrile and the addition of NaCl. Purification methodologies of these extracts varied. Solid Phase Extraction (SPE) such as Oasis HLB catridges [Mottier et al., 2006] or Molecular imprinted (MIPs) SPE cartridges [Mohamed et al., 2008] were used. In some methods the samples were just filtered before injection and satisfactory results were still achieved [Xia et al., 2006, Daeseleire et al., 2000, Cronly et al., 2009]. Methods for the analysis of these compounds in plasma are varied in their extraction protocols. Extraction solutions such as aqueous buffer [Aerts et al., 1991], NaCl/Potassium phosphate buffer (Fraselle et al., 2007) and acetonitrile [Thompson et al., 2009, Cronly et al., 2009 (b)] have been used in the extraction of NMZs from plasma. SPE catridges of Extrelut [Aerts et al., 1991 and Chromabond XTR [Fraselle et al., 2007] have been used in the sample purification in this matrix. Cronly et al. omits the use of SPE and clean up is achieved by purifying samples with the use of a hexane wash step and filtering before injection.

While suitable methods now exist for monitoring these analytes, no suitable methods in matrices such as milk and honey exist for the comprehensive confirmatory analysis of NMZs. The CRL for NMZs has suggested that honey be tested to ascertain any possible misuse and if non-compliant results are found then this matrix should be included in monitoring plans. They also state that countries with high milk production should also analyse for these analytes in milk as their possible misuse in this matrix cannot be discounted. From investigation of literature only one method could be found that allows for the analysis of NMZs in honey. Zhou et al., 2007 published a method for the analysis of 5 NMZs in honey by HPLC-UV. Samples were extracted with ethyl acetate and evaporated. The residue containing the NMZs was dissolved in ethyl acetate–hexane and subjected to solid-phase extraction cleanup by amino extraction columns. The eluent was evaporated, reconstituted and injected onto the column.

In relation to the analysis of nitroimidazoles in milk there are a limited number of published methods available [Ortelli et al., 2009; Stolker et al., 2008, Thompson et al., 2007]. These methods are all screening methods using either optical biosensor [Thompson et al., 2007] or accurate mass instruments [Ortelli et al., 2009; Stolker et al., 2008]. From investigation of literature there are no methods for the confirmatory analysis of NMZs in milk at the levels desired.

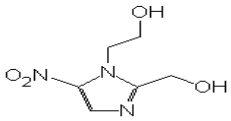
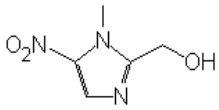
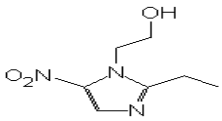
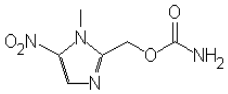
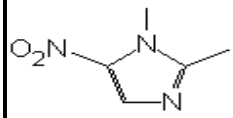
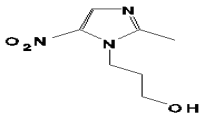
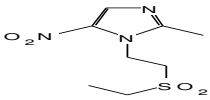
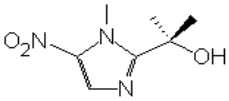
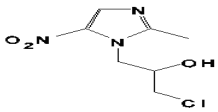
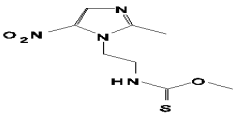
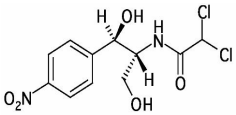
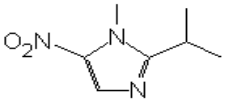
1.2. Chloramphenicol

Chloramphenicol (CAP) is a broad spectrum bacteriostatic antibiotic that exerts its effect by inhibiting bacterial protein synthesis. The structure of CAP is seen in table 1. Research carried out on CAP has shown that it has adverse toxicological affects in human such as aplastic anaemia or grey-syndrome. Due to these health concerns it has been classified as an A6 compound in annex IV of council regulation 2377/90 which means it is prohibited for use in food producing species. CAP has been issued a minimum required performance limit (MRPL) of 0.3 ng mL^{-1} which means all methods should be able to at least see to this level. As a result of this low level; CAP is often analysed in single analyte methods [Rejtharova et al., 2009; Ronning et al., 2006] although some multi amphenicol methods do exist [Zhang et al., 2008; Shen et al., 2009].

A common trend in analysis of CAP in any matrix is the use of an SPE clean-up. Common cartridge chemistries used in the analysis of CAP are Oasis HLB [Shen et al., 2009], Oasis MCX [Zhang et al., 2008] and the selective technique of Molecular Imprinted Polymers (MIPs) [Rejtharova et al., 2009; Boyd et al., 2007] An investigation into the methods used to analyse for CAP found that it is often analysed singly in several matrices including milk and honey. Ronning et al., 2006 developed a method that anaylse for CAP residues in meat, seafood, egg, honey, milk, plasma and urine with liquid chromatography–tandem mass spectrometry. Samples were extracted with acetonitrile and chloroform was added to remove water. Extracts were then evaporated to dryness, reconstituted and filtered before injection. Rejtharova et al., 2009 described a method for the analysis of CAP in urine, feed water, milk and honey samples by GC-MS-NCI using molecular imprinted polymer clean-up.

On examining published literature it is quite clear that the analysis of NMZs in milk and honey has not been examined to date. No confirmatory methods could be found for the analysis of these analytes in the matrices of honey and milk. With growing concerns from China about the use of NMZs in honey the CRL having recommended that this matrix be examined. The CRL have also recommended that countries with high milk production should analyse for these compounds in milk. Therefore there is a need for a comprehensive method to allow for the confirmatory analysis of both these matrices. In addition to this, while the analysis of CAP in all matrices is being performed it is often performed using single analyte methods which contain time consuming SPE clean-up steps.

Table 1: Molecular Structures, Retention time (Rt), Precursor and Product ions, Collision energies and typical ion ratios for all 11 analytes

Compound	Structure	Rt	Precursor (M/z)	Product (M/z)	Collision Energy	Typical Ion Ratio
MNZ-OH 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole		1.34	188	123	17	0.71
				126	23	
HMMNI 2-hydroxymethyl-1-methyl-5-nitroimidazole		1.77	158	110	20	0.73
				140	17	
Metronidazole (MNZ) 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole		2.11	172	82	33	0.70
				128	19	
Ronidazole (RNZ) 1-methyl-2-[(carbamoyloxy) methyl]-5-nitroimidazole		2.38	201	140	17	0.33
				55	29	
Dimetridazole (DMZ) 1, 2-dimethyl-5-nitroimidazole		3.05	142	96	21	0.30
				81	31	
Ternidazole (TRZ) 2-Methyl-5-nitroimidazole-1-propanol; 3-(2-Methyl-5-nitroimidazol-1-yl)propan-1-ol		3.69	186	128	19	0.46
				82	37	
Tinidazole (TNZ) 1-(2-ethylsulfonylethyl)-2-methyl-5-nitroimidazole		3.97	248	121	23	0.19
				82	47	
IPZ-OH 1-methyl-2-(2′-hydroxyisopropyl)-5-nitroimidazole		4.09	186	168	19	0.50
				122	27	
Ornidazole (ORZ) 1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole		4.11	220	128	23	0.58
				82	39	
Carnidazole (CRZ) 1-(2-ethylcarbamothioic acid O-methyl ester)-2-methyl-5-nitroimidazole		4.27	245	118	21	0.17
				75	43	
Chloramphenicol (CAP) 2,2-dichloro-N-[(1R,2R)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]acetamide		4.25	321	257	-16	0.90
				152	-12	
Ipronidazole (IPZ) 2-isopropyl-1-methyl-5-nitroimidazole		4.32	170	124	25	0.65
				109	33	

From literature investigation no method was found that was capable of analysing for CAP and the eleven NMZs listed in this paper. The sample preparation described in this study is less time consuming than previously published methods. The milk method has a sample size of 1ml which allows for fast extraction times. The majority of methods for the analysis of CAP incorporate the use of an SPE clean up. The method presented here omits this step and still achieves satisfactory results.

2. Materials and Methods

2.1 Materials and Reagents

CAP and CAP-d5 were purchased from Sigma (St. Louis, MO) and Dr. Ehrenstorfer GmbH(Germany) respectively. CNZ, TNZ, TRZ, ORZ were provided by the CRL (BVL, Berlin, Germany). RNZ and DMZ were purchased from Sigma (St. Louis, MO) and MNZ, IPZ, IPZ-OH, HMMNI, MNZ-OH, HMMNI-d3, MNZ-OH-d2, DMZ-d3, RNZ-d3 were purchased from WITEGA Laboratorien (Berlin, Germany). Water is of LC-MS grade (Fluka). All other solvents were of LC grade and purchased from Reagecon (Clare, Ireland). Sodium chloride was AnalaR grade and purchased from VWR (Poole, England). Individual stock standards of each analyte at 1mg ml^{-1} in ethanol were prepared and stored at 4˚C for 1 year. Individual intermediate standard solutions (10,000 and 100 ng ml^{-1}) in methanol were prepared. Two working standards solutions (mixture of analytes) were prepared in methanol containing all NMZs at levels of 300ng ml^{-1} and 100ng ml^{-1} respectively (for honey) and CAP at levels of 30ng ml^{-1} and 10ng ml^{-1} respectively(for milk). Deuterated standards were prepared similarly except the mixed standard contained 200ng ml^{-1} deuterated NMZs and 20ng ml^{-1} deuterated CAP.

2.2 LC-MS/MS Instrumentation

The LC-MS/MS system is an AB Sciex Triple Quad 5500 couple to Schimadzu UFLC XR LC system. The instrument is controlled by Analyst Software 1.5 and operated in positive and negative electrospray ionisation (ESI +/-). Separation was achieved using a 100x2mm, 1.8 micron particle size Zorbax Eclipse Plus C18 column supplied by Agilent Technologies (Santa Clara, CA). The column oven temperature was set at 45 ºC. The chromatographic separation was achieved using gradient mode consisting of water acidified with 0.1% acetic acid (mobile phase A) and acetonitrile acidified with 0.1% acetic acid at flow rate 0.5ml min^{-1}. The gradient is as follows; 95% A for the first 1.5 min. Then this changes to 5% A from 1.5-3.0 min and maintained for 2.0 min. The conditions then return to the initial 95% A in 1.0 min (5-6min) and remain the same till the end of the run of 9.0 min. A divert valve is utilised to help remove any matrix impurities from entering the MS/MS. The LC flow is diverted for the first minute and the last three minutes of the method. The ionisation mode used was positive electrospray ionisation for the NMZs and negative electrospray ionisation for CAP. The MS/MS method was segmented in order to obtain enough data points on each peak. The first three minutes is run in positive mode and from then on it is run with positive and negative switching. A cone temperature of ºC with a spray voltage of V was used to produce parent to product ions. The individual precursor and products ions for each analyte with their respective collision energies are listed in table 1.

2.3 Milk and Honey Samples

Milk and honey were obtained and stored at -20 ºC. Portions of these samples were analysed and those found to contain no detectable residues of the analytes of interest were used as blanks for the validation study. Chromatograms of blank milk and honey samples can be seen in figure 1 and 2. For the day four validation studies of variability due to matrix variances a wide range of milk and honey samples were obtained. Milk samples comprised of high fat, low fat, organic milk and also milk with added extra calcium, folic acid and vitamins A, B, D and E, a sample of goat's milk was also examined. Honey samples comprised of the following types Kapetanios Pure Greek; Rowse Australian Eucalyptus; Capilano Australian Organic Blended; Tropical Forest Ltd. Ethiopian Forest; Marks and Spencer's New Zealand Clover; Famille Michaud-Lavender Honey, Provence, France; New Zealand Manuka Honey, Irish; Irish Honeycomb, Dublin; De Rit Blended Flower.

2.4 Methods

2.4.1. Milk Extraction

Milk (1mL) was pipetted into polypropylene centrifuge tubes (15mL). These were fortified with mixed internal standard (30 µL) which corresponded to 6 ng mL^{-1} of deuterated NMZs and 0.6 ng mL^{-1} deuterated CAP. Acetonitrile (2mL) was added and vortexed. NaCl (0.5g) was added to this slurry which was shaken (30 secs) and then centrifuged (4350 x g for 10min). The top organic layer from each sample was then transferred to amber vials (5mL) and evaporated (50ºC) to dryness under a stream of nitrogen. The extracts were reconstituted in Water: Acetonitrile (95:5, 200µL) and filtered through 0.2µm PVDF syringe filters. An aliquot (10µL) was injected onto the LC column.

2.4.2. Honey Extraction

Honey (3 g) was weighed into polypropylene centrifuge tubes (50 mL). These were then placed in an oven at 50 ºC for 30 min to soften. The samples were then fortified with mixed internal standard (50 µL) which correspond to 2 µg kg^{-1} of deuterated NMZs and 0.2 µg kg^{-1} deuterated CAP. Water (5ml) was then added to each sample and these are then placed back in the oven for a further 10 min. The samples were then thoroughly vortexed until the honey was fully dissolved in the water. To this acetonitrile (10 mL) was added and the tubes were vortexed (20secs). NaCl (2 g) was added to this slurry which was then shaken (30secs) and centrifuged (4350 x g for 10min). The top organic layers were then transferred to polypropylene tubes (15 mL) and evaporated (50ºC) to 6mL under nitrogen. Hexane (5 mL) was added and this was vortexed (30secs). The hexane layer was then discarded and the extracts were evaporated to dryness at 50ºC under a nitrogen stream. They were then reconstituted in Water:ACN (200 µL of 95:5 and filtered through 0.2 µm PVDF syringe filters. An aliquot (10 µL) was injected onto the LC column.

2.5 Matrix Extracted Calibration Curves

Quantitation was carried out using matrix-matched calibration curves. Blank honey and milk samples were used. These samples were fortified with mixed working standard and submitted to the full extraction procedure of the method. A matrix matched calibration curve is performed with every batch. Six samples are fortified with internal standard and mixed working standard for a calibration range of 0 to 20 ng mL^{-1} (µg kg^{-1} Honey) for the NMZs and a range of 0-2 ng mL^{-1} (µg kg^{-1} Honey) for CAP. Calibration curves were prepared by plotting the response factor (the ratio of peak area analyte over peak area of internal standard) against analyte concentration. Seven deuterated internal standards are used; d3- DMZ, d3-RNZ, d3-HMMNI, d2-MNZ-OH, d3-IPZ, d3-IPZ-OH and d5-CAP. For those compounds with no deuterated analogues; MNZ, TRZ, TNZ, ORZ and CRZ, d3-HMMNI is used as an internal standard. For each analyte calibration curves were linear in the given range with a correlation coefficient of at least 0.99.

2.6 Method validation

The LC–MS/MS method was validated according to 2002/657/EC guidelines. The same validation protocol was used for both the honey and milk matrix. LC–MS/MS identification criteria were verified throughout the validation study by monitoring relative retention times, ion recognition (signal-to-noise ratio (S/N)) and relative ion intensities. LC-MS/MS identification criteria as set out in the legislation were verified throughout the validation of the method.

Several method validation parameters were determined including linearity, specificity, recovery, precision (repeatability and within-laboratory reproducibility) and analytical limits (decision limit CCα and detection capability CCβ). Specificity was determined by analysing 10 different blank milk and honey samples sourced from different suppliers. No interfering peaks were observed at the retention time for any of the transitions. This allows for clear identification and quantification of all analytes. To investigate the linearity of the method, matrix-matched calibration curves were prepared and run with each of the validation batches to give 6 point calibration curves ranging from 0 to 20µg L^{-1} / µg kg^{-1} for the NMZs and 0 to 2µg L^{-1} / µg kg^{-1} for CAP.

Since no certified reference materials were available for the analytes and matrices of interest, the recovery from fortified negative samples was measured as an alternative to trueness. The recovery and precision were determined through the analysis of negative milk and honey samples fortified in six replicates at 1, 1.5 and 2 times the RL and the MRPL of 3 and 0.3 µg L^{-1} / µg kg^{-1} for NMZs and CAP respectively. Six replicate test portions at each of the three fortification levels (n = 18) were analysed on three separate days over a period of two weeks. Samples were fortified with NMZs at 3, 4.5 and 6 µg L^{-1} / µg $kg^{-1}$and with CAP at 0.3, 0.45 and 0.6 µg L^{-1} / µg kg^{-1} by adding mix working standard solution (30, 45 and 60 µL) and analysed. To determine any matrix effects caused by biological variations arising from various milk and honey samples a fourth day analysis was carried out. For each matrix two sets of ten different samples were analysed. The first set was fortified with only internal standard, and the second set was fortified with both internal standard and with the analytes at a concentration equivalent to 4.5µg L^{-1} / µg $kg^{-1}$of NMZs and 0.45µg L^{-1} / µg kg^{-1} of CAP. From these four separate validation days an estimation of recovery, precision (repeatability and within-laboratory reproducibility) and analytical limits (decision limit CCα, and detection capability CCβ) were determined.

3. Results and Discussion

3.1. Method Optimisation

The method was developed to provide confirmatory data for the analysis of CAP and 11 NMZs in milk and honey. The method was developed from an existing method used in the lab to analyse for NMZs in plasma and eggs [Cronly et al., 2009(a), (b)]. The LC-MS/MS instrument used for this method was different than the one used previously so fragmentation conditions were investigated and collision energies were optimised for each individual compound to give best response. For a method to be deemed confirmatory under Commission Decision 2002/657/EC it must yield 4 identification points. In this method a precursor

ion (parent mass) and two product ions (corresponding to strong and weak ion) are monitored for each analyte (Table 1). This yields 4 identification points (1 for the parent ion and 1.5 for each daughter ion) hence it can be deemed a confirmatory method. In addition to this relative retention times and ion ratios are tracked for each compound and ensured that they are within acceptable ranges stated in EC 2002/657. The LC gradient was also optimised in order to have a quick run time but also have enough data points for each peak. For a method to achieve reliable quantitation each analyte peak should have at least 10-12 data points. As this method involved positive and negative ionisation switching the MS/MS method had to be segmented. This along with altering the LC gradient allowed for the analysis of all 12 analytes in a complete run time of less than 9 minutes.

Table 2. Validation Results for Milk and Honey; Coefficient of Variance (%CV), Accuracy, Decision Limits (CCα) and Detection Capabilities (CCβ), Measurement Uncertainty (MU) and Correlation Coefficients (R^2)

3.2. Validation

Validation is carried out in accordance with the procedures outlined in Commission Decision 2002/657/EC covering specificity, calibration curve linearity, accuracy, precision, decision limits (CCα), decision capability (CCβ) and measurement uncertainty. Results are seen in table 2 for these criteria. The ruggedness of the method is demonstrated on an ongoing basis through the use of it to analyse National Residue Control Plan milk and honey samples in Ireland. The criteria of relative retention times (RRT) and ion ratios were monitored for all analytes in the four validations days. The values identified for these were all within European requirements. The RRT tolerance of 2.5% was adhered to when standards were compared to samples in the validation runs. Two transition ions were monitored for each of the twelve analytes. The most intense was used for quantitation. All ion ratios of samples were within tolerances as set out by European criteria when compared with standards used during validation.

Analytes	CV%		Accuracy %		CCα µg kg-1		CCβ µg kg-1		MU %		R^2 Value
	Milk	Honey	Milk	Honey	Milk	Honey	Milk	Honey	Milk	Honey	
MNZ-OH	4.5	4.1	92.0	103.8	0.43	0.39	0.73	0.67	27	25	>0.99
HMMNI	4.1	4.6	94.5	104.2	0.42	0.42	0.71	0.71	27	29	>0.99
MNZ	7.8	6.7	92.6	108.9	0.65	0.72	1.10	1.22	30	51	>0.99
RNZ	4.3	3.5	94.3	102.4	0.41	0.38	0.70	0.66	23	24	>0.99
DMZ	9.2	9.4	96.2	94.7	1.08	0.73	1.83	1.24	40	42	>0.99
TRZ	5.4	9.0	93.9	102.0	0.53	0.78	0.91	1.34	23	56	>0.99
TNZ	15.3	12.4	90.5	104.4	1.55	1.16	2.64	1.98	61	64	>0.99
IPZ-OH	4.8	6.2	94.2	100.8	0.46	0.64	0.78	1.10	20	39	>0.99
ORZ	11.4	11.3	90.8	100.7	1.08	1.00	1.85	1.71	69	81	>0.99
CRZ	10.8	10.7	91.7	101.0	1.23	0.95	2.09	1.62	40	90	>0.99
CAP	7.5	8.0	95.1	100.7	0.07	0.08	0.11	0.13	32	31	>0.99
IPZ	4.7	4.3	95	97.8	0.52	0.40	0.88	0.68	25	35	>0.99

3.2.1 Specificity

The technique of LC-MS/MS itself offers a great deal of specificity and selectivity. To establish the specificity and selectivity of the method blank milk and honey samples and samples fortified with all 12 analytes were analysed over the 3 validation days. On the fourth day 10 different types of milk and honey samples were also analysed. Blank samples showed no interfering peaks in the area of interest for any of the analytes. Chromatograms of blank milk and honey and milk and honey fortified at the RL and MRPL for each of the analytes are seen in figure 1 and 2.

3.2.2 Linearity

The linearity of the chromatographic response was tested with matrix matched calibration curves using six calibration points in the range of 0-20 µg L^{-1} / µg kg^{-1} NMZs and 0-2.0 µg L^{-1} / µg $kg^{-1}$for CAP. The regression coefficients for all the analytes on each of the validation days in both matrices were greater than 0.99.

3.2.3 *Accuracy/Trueness*

The accuracy (trueness) of the method was determined by fortifying 6 replicate milk and honey samples at 1, 1.5 and 2 times the analytes respective RL or MRPL on three separate days. Mean corrected recovery (n=6) of the analytes, determined in the three separate validation batches are shown in table 2 range between 90.8 and 108.9% for the twelve analytes in both matrices. No absolute recovery was determined as the use of internal standards means that each sample is individually corrected for.

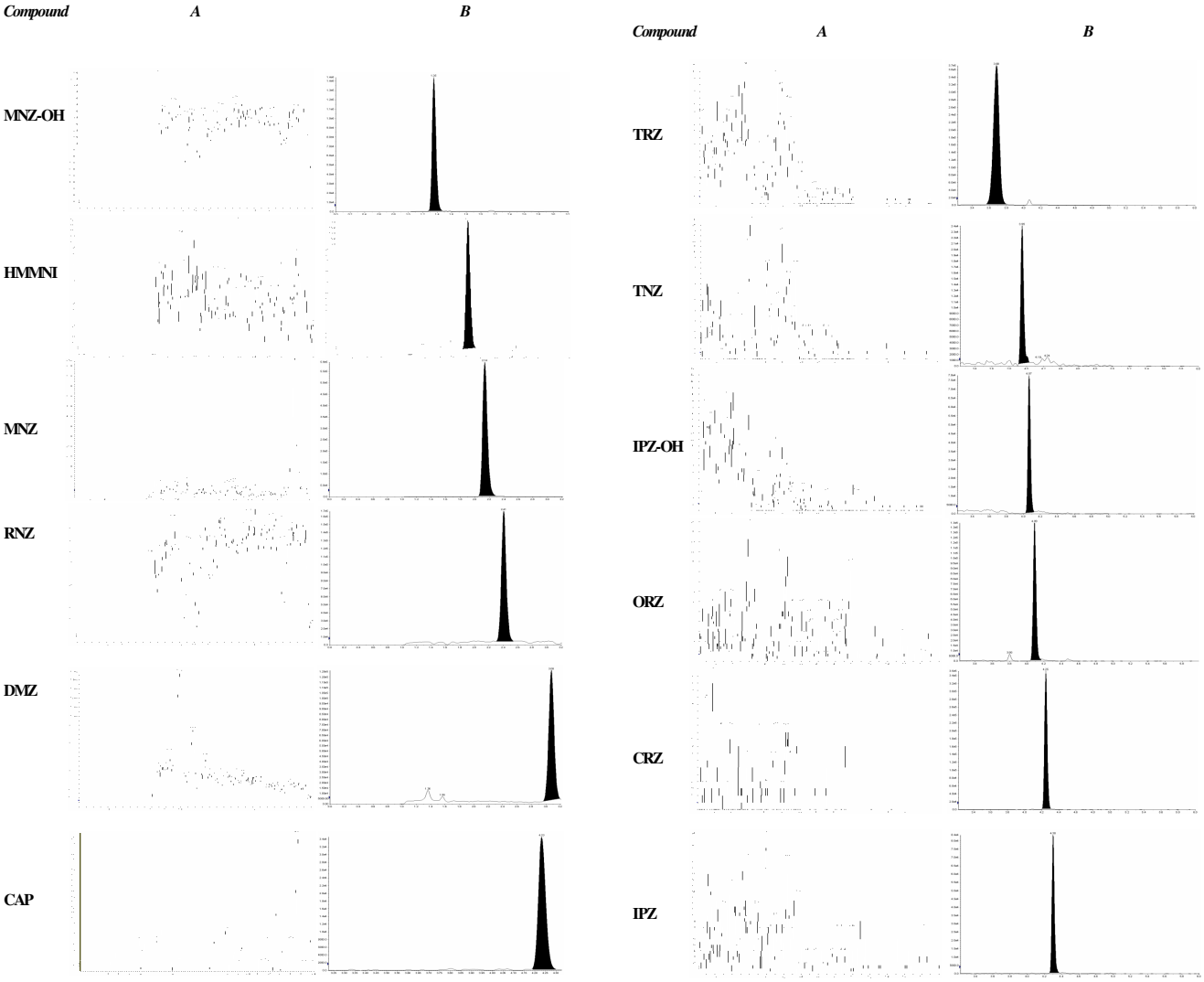


Figure 1. Chromatogram of blank milk (A) and milk (1ml) fortified at 2.5 for NMZs and 0.25 for CAP (B)

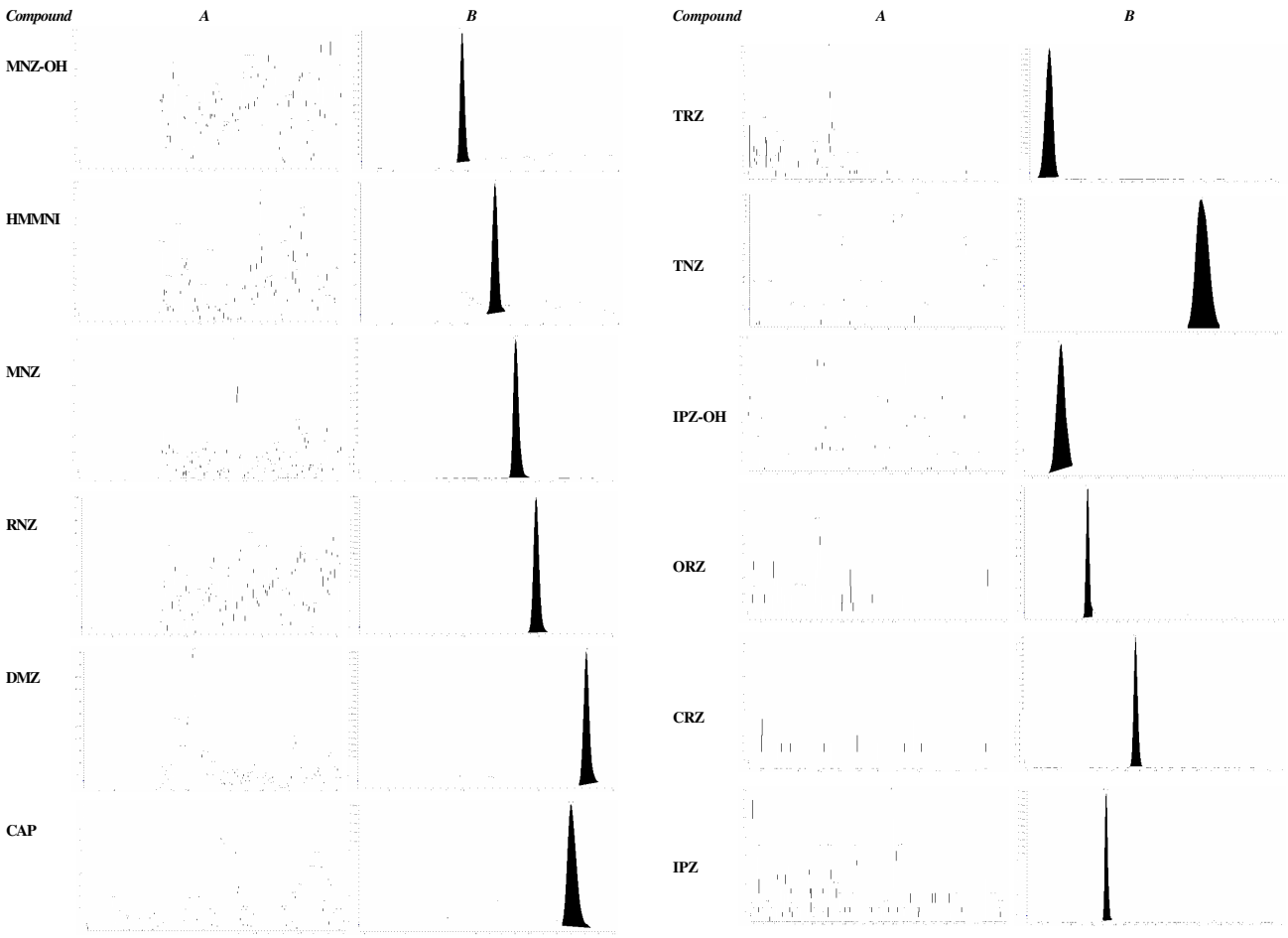


Figure 2. Chromatogram of blank honey(A) and honey (3g) fortified at 2.5 for NMZs and 0.25 for CAP (B**)**

3.2.4. Precision

Satisfactory values for inter-assay precision expressed as %CV values for the within lab reproducibility (table 2) were achieved for all analytes in both matrices. According to Commission Decision 2002/657/EC this coefficient of variance for the repeated analysis of fortified material under reproducible conditions shall not exceed the level calculated by the Horwitz equation. For a concentration of 100 µg L^{-1} / µg kg^{-1} this equation gives a value of 23%. However when concentrations go under this value the equation gives unacceptably high results. Therefore its stated in Commission Decision 2002/657/EC that %CV should be kept as low as possible. Results achieved range from 3.5 to 15.3% for all analytes in both matrices. These acceptable results can be attributed to the availability of 7 deuterated analogues to use as internal standards. These also worked well for compounds with no deuterated internal standards such as CNZ, ORZ, TRZ and TNZ.

3.2.5. CCα and CCβ

CCα is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. For prohibited substances an α value equal to 1 % is applied. CCβ is the smallest content of the substance that may be detected, identified and quantified in a sample, with a statistical certainty of 1-β, where $\beta = 5\ \%$. CCα and CCβ were calculated using the calibration curve procedure in accordance with ISO 11843. After identification, the signal is plotted against the added concentration. The corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept equals the CCα. CCα values of for the 12 compounds in honey and milk are listed in Table 2 and are all below 1.55 µg L^{-1} / µg $kg^{-1}$for the NMZs and below 0.08 µg L^{-1} / µg kg^{-1} for CAP. CCβ is the concentration corresponding to the signal at CCα + 1.64 times the standard error of the intercept (i.e. the intercept + 3.97 times the standard error of the intercept). CCβ values for all compounds in honey and milk are listed in table 2

and are all below the RL of 3ng mL^{-1} and MRPL of 0.3µg kg^{-1} for NMZs and CAP respectively.

3.2.6. Measurement Uncertainty

The measurement uncertainty (MU) was estimated by taking into account the within laboratory reproducibility over days 1, 2 and 3 as well as considering the repeatability on day 4 due to matrix effects caused by various honey and milk samples. These two variability's were combined and multiplied by a coverage factor of three to give an overall figure for the MU. This approach of using the within laboratory reproducibility as a good estimator of measurement of uncertainty is taken from the SANCO/2004/2726rev1 document. It recommends using the within laboratory reproducibility and using a coverage factor of 2.33 to estimate expanded uncertainty, however it was felt that not all the environmental factors that could be varied over the course of the validation were examined. Therefore a coverage factor of 2.33 may underestimate the true uncertainty of the method and instead a value of 3 was chosen to give a more realistic value for the true uncertainty. Values for MU are seen in Table 2 and lie between 23 and 69% for all compounds in milk and between 24 and 90% for all analytes in honey.

Higher MUs are seen in milk for some compounds with no deuterated analogues to use as internal standards which is expected. Honey results in general display higher MUs. High MU estimates are again seen for some of the compounds with no deuterated analogues to use as internal standards in particular ORZ (81%) and CRZ (90%). Their MU estimates in honey are the highest due to large values for reproducibility due to matrix as a result of a variation between matrices used in the day 4 experiment and the lack of suitable internal standards.LC–MS/MS identification criteria were verified throughout the validation study by monitoring relative retention times and relative ion intensities. LC-MS/MS identification criteria as set out in the Commission Decision 2002/657 were verified throughout the validation of the method.

4. Conclusions

The objective of this work was to develop a rapid multi-class multi-residue confirmatory method capable of identifying, confirming and quantifying NMZ compounds and CAP in milk and honey at µg L^{-1} and µg kg^{-1} levels and to validate according to the requirements in Commission Decision 2002/657/EC. This was successfully completed.

The method can be considered as rapid, as it utilises an efficient extraction protocol without the use of SPE. It also utilises chromatography which separates all analytes in a total run time of only 9 minutes. The method includes the confirmatory analysis of CAP and 11 NMZs in milk and honey which has not been seen before.

The obtained data fulfills the requirements laid down in Commission Decision 2002/657/EC and allows the calculation of all relevant performance characteristics. This study shows that the developed method meets the required sensitivities of 3 µg L^{-1} / µg kg^{-1} for NMZs and 0.3µg L^{-1} / µg $kg^{-1}$for CAP which are the RL and MRPL used for these compounds. The CCα and CCβ values determined for each analyte are lower than this level. The method performs very well in terms of accuracy and repeatability for each of the analytes due to the utilisation of seven different deuterated internal standards. The values achieved for accuracy, %CV and measurement of uncertainty all fall within acceptable ranges. The applicability of the method for use on various types of milk and honey samples was demonstrated by the satisfactory results obtained from the Day 4 analysis of different species.The reduced number of analytical steps within the method makes it very amenable for high through-put regulatory monitoring of these compounds.

From examination of published literature no method was found that was capable of the thorough analysis of CAP and eleven NMZs in milk and honey. Methods published on these matrices analysed at most seven analytes and in the case of CAP often analysed as a single analyte method. The method developed in this study allows for improvement on any existing method as it allows for the analysis of an increased number of analytes in matrices that have been previously overlooked. It also allows for reduced sample preparation times as SPE clean-up has been omitted. In the case of the extraction protocol for milk time and solvent usage is greatly reduced compared to other published methods as a result of reduced sample size of 1mL.

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