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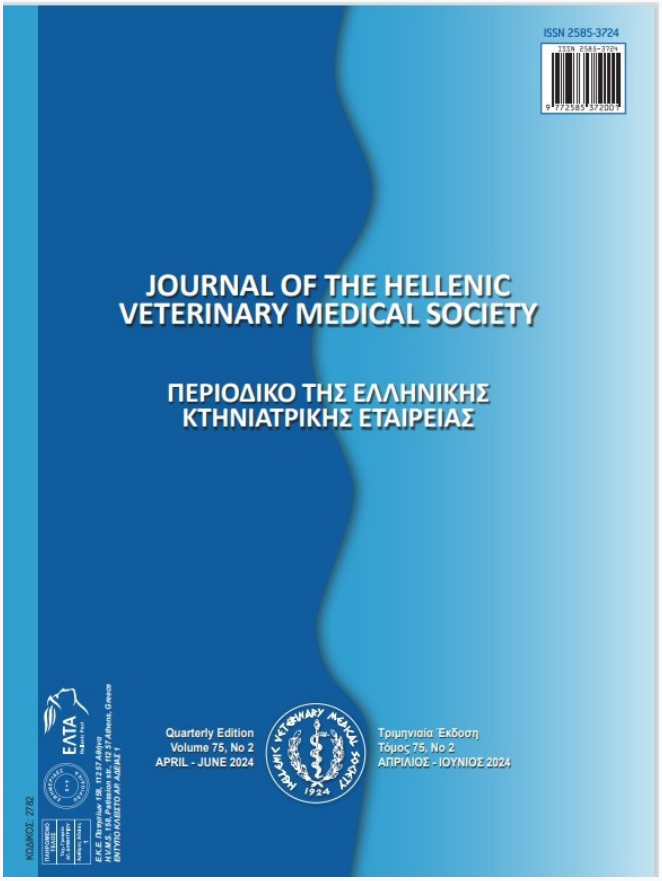

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Determination of Urea Content in Dairy Cattle Feed by High Performance Liquid Chromatography via Refractive Index Detector: Method Optimization, Validation and Comparison with Spectro-Colorimetric Method

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ABSTRACT: The aim of this study was to analyze the urea content of Dairy Cattle Feed (DCF) using High Performance Liquid Chromatography via Refractive Index Detector (HPLC-RID), without derivatization, along with spectro-colorimetric method that is the official control method of European Union (EU). Towards that goal, the analysis procedure for the method was established, method optimization and validation were carried out by spiking urea at 2% level to DCF, which is the upper limit allowed for use in ruminant feeds in Türkiye. In method validation; R^2: 0.9997 coefficient of determination at 25-100000 mg/kg linear range, 75 mg/kg limit of detection (LOD) and 250 mg/kg limit of quantification (LOQ), 1.08% repeatability RSD, 1.84% reproducibility RSD and 95.10% recovery were obtained. The applicability of the method was proven at 25-100000 mg/kg linear range with real samples by spiking 2% urea in Urea-Free Dairy Cattle Feed (UF-DCF), Low Urea Dairy Cattle Feed (LU-DCF), and High Urea Dairy Cattle Feed (HU-DCF). The presence of urea in UF-DCF was detected with the spectro-colorimetric (at 420 and 435 nm wavelength) method, while urea in UF-DCF was not detected by the in-house HPLC-RID method, ($P<0.05$). The amount of urea in LU-DCF without spiking urea was detected by the same level as HPLC-RID method and the SC-435 method ($P>0.05$). For HU-DCF blank samples, higher urea amounts were detected with the in-house HPLC-RID method as compared to the spectro-colorimetric methods used in the study ($P<0.05$). In DCF spiked with 2.0% urea, the urea amount found in the in-house HPLC-RID method were higher than that of the spectro-colorimetric method ($P<0.05$). As a result, the in-house HPLC-RID method that is developed in this study has shown great promise to be a potential, applicable and valid method for determining the urea amount in DCF.

Keywords: Feed additives; dairy cattle feed; urea; HPLC; spectrophotometry

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INTRODUCTION

Urea, as a non-proteinous and nitrogenous compounds, has been used for more than 100 years as a protein source to nurture ruminants. (Kertz, 2010; Patra and Aschenbach, 2018). By adding urea into compound feeds, pulps, hay, and silages, the protein content of feed can be increased (Pibarot and Pilard, 2012; Anitha et al., 2022). In European Union Commission Implementation Regulation (EU) No 839/2012, the maximum urea level allowed to put into ruminant feeds was limited at 8800 mg/kg concentration calculated based on 12 % humidity in feed. (Anonymous, 2012). In Türkiye, the legal legislation regarding the use of urea in feeds, it can be use maximum 2% in rations for only ruminant that have completed rumen development (Anonymous, 2011).

In the labs, the presence of urea in the feeds was determined not only via qualitative tests (Chauhan Mahipalsinh et al., 2017), but also quantitative tests (Giraldo and Rivas, 2017; Phonchai et al., 2020). Standards used in quantitative urea determination studies are complied with national and international organizations. Many countries, especially EU countries and Türkiye, use spectro-colorimetric method in determining urea in feeds (Anonymous, 1970; Anonymous, 2008; Anonymous, 2009; Anonymous, 2017).

In recent years, there have been many studies towards determining urea in different matrixes with High Performance Liquid Chromatography (HPLC). Various fermented foods and beverages (Matsudo and Sasaki, 1995), human and animal urine and wine (Clark et al., 2007), milk and milk powder (Dai et al., 2010; 2012), Chinese yellow rice wine (Wang et al., 2014), liquid fertilizers (Hojjatie and Abrams, 2015), canned foods (Zeng et al., 2015) and creams (Wang et al., 2016) were subjected to urea analysis. It was determined that there are some challenges in determining the urea accurately since the extraction cannot be performed effectively if samples are oily and opaque, meaning that other substances in the samples might get into a reaction with urea (Czauderna and Kowalczyk, 2012; Hojjatie and Abrams, 2015). With reverse or normal phase chromatography, urea cannot be separated from free amino acids or bioamins (Koebel and Elsener, 1995).

By utilizing HPLC for determining urea in feeds, the very first study was performed on pet food by Pibarot and Pilard (2012). Also, it was reported that free amino acids react with 4-dimethylaminobenzaldehyde (4-DMAB) and can be absorbed in 435 nm wavelength. The researchers determined the urea in animal feeds higher with spectro-colorimetric method compared to enzymatic and liquid chromatography ultraviolet-visible diode array detection (LC/UV-DAD) methods, and informed that free amino acid peaks aligned after the urea peak. Determining urea with HPLC method was carried out in animal feeds (Wegh et al., 2018), yeast-based food and feed yeast (Flannelly et al., 2019), compound feed, pet food, and yeast (Krämer et al., 2021).

It is inevitable that specific studies will be carried out in to determine the urea analysis by HPLC in different feed and feed substances. To the best of our knowledge, up until now, there have been no studies aimed at determining urea in Dairy Cattle Feed (DCF) with High Performance Liquid Chromatography Refractive Index Detector (HPLC-RID). Therefore, this study aimed to develop a method for the analysis of DCF urea content with HPLC-RID without derivatization process used in the previous studies. The results obtained in this study were compared with the spectro-colorimetric method, suggesting that the method developed in this study can be used in routine analyses was proposed.

MATERIALS AND METHODS

Materials

The feed materials of the study consist of DCF obtained from compound feed mills in Bursa region of Türkiye in 2022 production year. For sample taking, approximately 2 kg of each sample was selected; 4 Urea-Free Dairy Cattle Feed (UF-DCF), 4 Low Urea Dairy Cattle Feed (LU-DCF), and 3 High Urea Dairy Cattle Feed (HU-DCF). The feed materials were ground in Fritsch Pulverisette 14 (Idar-Oberstein, Germany) lab mill at 1 mm sieve diameter and stored in laboratory conditions in glass jars.

High Performance Liquid Chromatography Refractive Index Detector (HPLC-RID) Method

In DCF, for the quantitative analysis of urea, in-house method, developed with HPLC-RID device with SIL-20A HT (Shimadzu, Japan) auto sampler and RID-10A (Shimadzu, Japan) Refractive Index Detector, was used. InertSustain NH_2 (5 µm, 250x4.6mm; GL Sciences Inc., Japan) column was used and it was conditioned at 25°C oven temperature. As mobile phase (90/10) acetonitrile/water (v/v) was used with 1 ml/min flow rate at 10 µl injection of samples. Total run time for HPLC-RID was 23 minutes. The water

used for the analysis was distilled in Elga Purelab Option-Q (United Kingdom) ultra-pure water (18 MΩ) system. In the preparation of urea standard solutions, 99.0-100.5% pure (Cat No: U5128, Sigma-Aldrich) urea was used. Mobile phase solution was prepared using 99.5% pure acetonitrile (Cat No: 439134, Sigma-Aldrich).

After the mobile phase (90/10) acetonitrile/water (v/v) was prepared, it was degassed in UCP-20 Ultrasonic Cleaner (LC Shop, Billerica, USA) ultrasonic water bath for half an hour. For urea stock solution, the urea was weighed with 1.001 g sensitiveness, it was transferred to 100 ml capped volumetric flask, the prepared mobile phase (90/10) acetonitrile/water (v/v) was added to ensure the urea dissolves. Then, the volumetric flask volume was filled with mobile phase solution, it was homogenized by vortexing with Shakers & Mixers (Heidolph, Germany) for 30 seconds. Urea standards at 0.0100, 0.0250, 0.0500, 0.100, and 0.125% concentration used in the calibration curve was prepared from the urea stock solution.

For the extraction of the sample, 5.0001 g of ground sample was weighed delicately into the 50 ml volumetric flask, after that 25 ml ultra-pure water was added. Volumetric flask cap was tightly closed, it was shaken for 10 minutes at 250 rpm/minute speed in the GFL 3018 mechanical shaker (LaborTechnik, Burgwedel, Germany). Having shaking was completed, the volumetric flask was filled to the volume of 50 ml with acetonitrile. Volumetric flask was tightly closed again and vortexed for 30 seconds. The content of the volumetric flask was filtered into a flask using a funnel with Whatman No:42 filtering paper. Using a 0.45 µm membrane filter injector, the filtrate was transferred to 2 ml amber colored vials. The vials were tightly closed and kept in a +4°C refrigerator until analysis.

To create the calibration curve, standard solutions containing 0.0125, 0.0250, 0.0500 and 0.100% urea were transferred to 2 ml amber colored vials using an injector with 0.45 µm membrane filter. The vials were tightly closed and stored in a +4°C refrigerator until analysis.

Spectro-Colorimetric Method

The method specified in AOAC 967.07 was used in determining urea with the spectro-colorimetric method in feed materials (Anonymous, 1970). In this method the urea in the feed material was extracted with water, it was clarified with Carez-I and Carez-II solutions. After adding equal amounts of 5 ml of 4-DMAB to the filtrate, at 420 nm and 435 nm wavelength absorbance value was measured deducting the optical density blank filtrate at the spectrophotometer (UV-Vis 1600) (Shimadzu, Japan). The concentrations of the colored compound of feed materials' filtrates that were produced by the reaction between 4-DMAB and filtrate were obtained by the calibration curve ranged wıth urea standard concentration at 50, 100, 200, 300 and 400 mg/l prepared from 5 g/l the urea stock solution.

Moisture Content

To fairly compare the urea contents, the moisture contents of feed samples were determined according to Commission Implementation Regulation (EU) No 839/2012 basis and it was adjusted according to 12% moisture content (corresponds to 88% dry substance). The moisture contents of the feed samples were determined according to Anonymous (2017).

In-House HPLC-RID Method Optimization

With HPLC-RID method to detect urea for 2.0% urea standard and 2.0% urea spiked UF-DCF urea concentrations, the mobile phase optimization of the urea analysis was carried out at (80/20), (85/15), (90/10), and (95/5) acetonitrile/water (v/v) mobile phases, according to the device conditions of the in-house method. In the optimization of sample extraction, 1.0, 2.5 and 5.0 g samples of UF-DCF were weighed. 2.0% urea was spiked at 12% moisture level to each of them and 10, 20, and 30 minutes shaking at 250 rpm/min. Each experimental process was repeated 5 times.

In-House HPLC-RID Method Validation

In the urea analysis with HPLC-RID method, the method validation was carried out according to the rules specified in Eurachem Guide 'The Fitness for Purpose of Analytical Methods (Anonymous, 2014). Calibration curve, linearity and measurement range, limit of detection (LOD) and limit of quantification (LOQ), precision (repeatability and reproducibility) and recovery were evaluated as parameters for the method validation.

Calibration curve, linearity and measurement range were determined based on the urea concentrations as 25, 50, 100, 500, 1000, 5000, 10000, 25000, 50000 and 100000 mg/l analyzed by in-house HPLC-RID method. To find out limit of detection (LOD) value, the lowest calibration curve point, which was, 25 mg/l, was

multiplied with 10. That value, 250 mg/l urea was injected in UF-DCF samples, and analysis of spiked samples were performed 10 repeats by utilizing in-house HPLC-RID method procedure. Standard deviation of the determined urea concentrations was calculated. Limit of detection (LOD) was calculated as three folds of the standard deviation found in the study. Limit of quantification (LOQ) was determined as 10 folds of the standard deviation calculated in the study.

Precision was determined by carrying out repeatability and reproducibility evaluations. For two parameters, 2.0% urea level, which is allowed to be put in ruminant feeds in Türkiye, was considered. For repeatability, UF-DCF was spiked with 2.0% urea, 6 independent studies were done in the same day. For reproducibility, UF-DCF was spiked with 2.0% urea, 2 independent studies in 6 different days were applied. The accuracy of the method was determined by calculating the recovery from repeatability and reproducibility since there were no certified reference materials (CRM).

Trial Groups

The moisture content of 10 different DCF and their urea contents were determined by the in-house method developed in the HPLC-RID. The determined urea contents were adjusted according to the 12% moisture content, and trial groups were classified as 3 UF-DCF, 4 LU-DCF and 3 HU-DCF. The feeds in the trial groups were spiked with 2.0% urea (to contain 2% urea at 12% humidity). Each feed was analyzed and urea contents were determined with in-house HPLC-RID, Spectro-Colorimetric-420 nm wavelength (SC-420) and Spectro-Colorimetric-435 nm wavelength (SC-435) methods in a way 5 parallel blank samples, 2.0% urea spiked samples and 2 parallel blank samples without spiking. The amount of urea in the blank sample was deducted from the urea content found in spiked feed samples, which was considered as recovery value for the method.

Statistical Analysis

In the analysis of urea by in-house HPLC-RID method, statistical analysis of method validation was performed according to the factorial experimental design. In-house HPLC-RID method optimization [sample amount (1.0, 2.5 and 5.0), shaking time (10, 20 and 30 minutes)] and trial group comparison [method (HPLC-RID, SC-420 and SC-435), feed urea level (UF-DCF, LU-DCF and HU-DCF)] were carried out according to four-factor factorial experimental design and 5 measurements were taken in each application. The 'Duncan's Multiple Range Test' was used to determine the significance level of the differences between the means. For the statistical analysis, data were evaluated using SAS version 8.3 (SAS Institute Inc., Cary,NC, USA, 1998).

RESULTS

Mobile Phase Optimization

In urea analysis with in-house HPLC-RID method, mobile phase [80/20; 85/15; 90/10; 95/5 acetonitrile/water (v/v)] optimization results were given in Table 1 and Figure 1. For [80/20; 85/15; 90/10 acetonitrile/water (v/v)] mobile phases, the retention times of urea were found as 5.17, 5.62, 6.62 minutes respectively. (95/5) acetonitrile/water (v/v) mobile phase was not evaluated since the retention time was further than 8.5 minute. In (85/15) and (90/10) acetonitrile/water (v/v) mobile phases, the amount of urea concentrations was close to each other.

Sample Extraction Optimization

The optimization results of sample extraction in urea analysis by in-house HPLC-RID method were given in Table 2. Sample amount did effect urea amount and recovery, 2.5 and 5.0 g sample amounts were determined to be more suitable for sample extraction optimization as compared to 1.0 g sample ($P<0.01$). In extractions with 2.5 and 5.0 g samples, urea contents were found as 2.03 and 2.00%, respectively. On the basis of the results, recoveries were calculated as 101.44 and 100.07%, respectively. It was indicating that sample amount did not influence the recovery rate ($P>0.01$). Shaking time and the interaction effect of sample amount x shaking time on

Table 1. Mobile phase optimization results for urea detection in HPLC-RID

Mobile phase Acetonitrile/ water (v/v)	Retention time (Minute)	Urea concentration (%)	
		2.0% Urea standard	UF-DCF+2.0% urea spike
80/20	5.17	1.69	1.82
85/15	5.62	1.85	1.99
90/10	6.62	2.06	2.08

UF-DCF: Urea-free dairy cattle feed

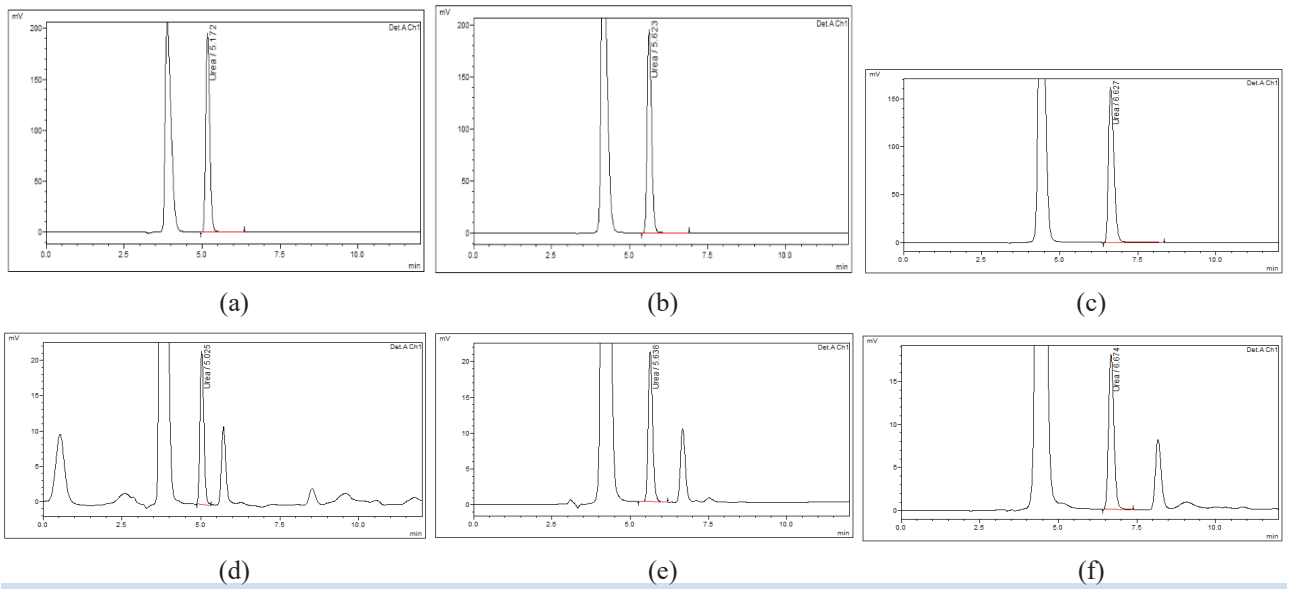


Figure 1. Chromatograms obtained in different mobile2 phases

a: (80/20) Acetonitrile/water (v/v) mobile phase for 2.0% urea standard, b: (85/15) Acetonitrile/water (v/v) mobile phase for 2.0% urea standard, c: (90/10) Acetonitrile/water (v/v) mobile phase for 2.0% urea standard, d: (80/20) Acetonitrile/water (v/v) mobile phase for UF-DCF+2.0% urea spiking, e: (85/15) Acetonitrile/water (v/v) mobile phase for UF-DCF+2.0% urea spiking, f: (90/10) Acetonitrile/water (v/v) mobile phase for UF-DCF+2.0% urea spiking
UF-DCF: Urea-free dairy cattle feed

Table 2. Optimization results of sample extraction

Source of variation	Treatment groups	Amount of urea (%)*	Recovery (%)*
Sample amount (g)	1.0	2.16±0.029[a]	107.99±0.007[a]
	2.5	2.03±0.012[b]	101.44±0.003[b]
	5.0	2.00±0.003[b]	100.07±0.001[b]
Shaking time (minute)	10	2.06±0.016	102.73±0.004
	20	2.08±0.032	103.90±0.008
	30	2.06±0.026	102.86±0.007
Sample amount × shaking time interaction effect		N.S.	N.S.

N.S: Not significant (P>0.01) *a, b: Within a column, means followed by different letter differ significantly (P<0.01).

the amount of urea and recovery was insignificant (P>0.01).

Method Validation

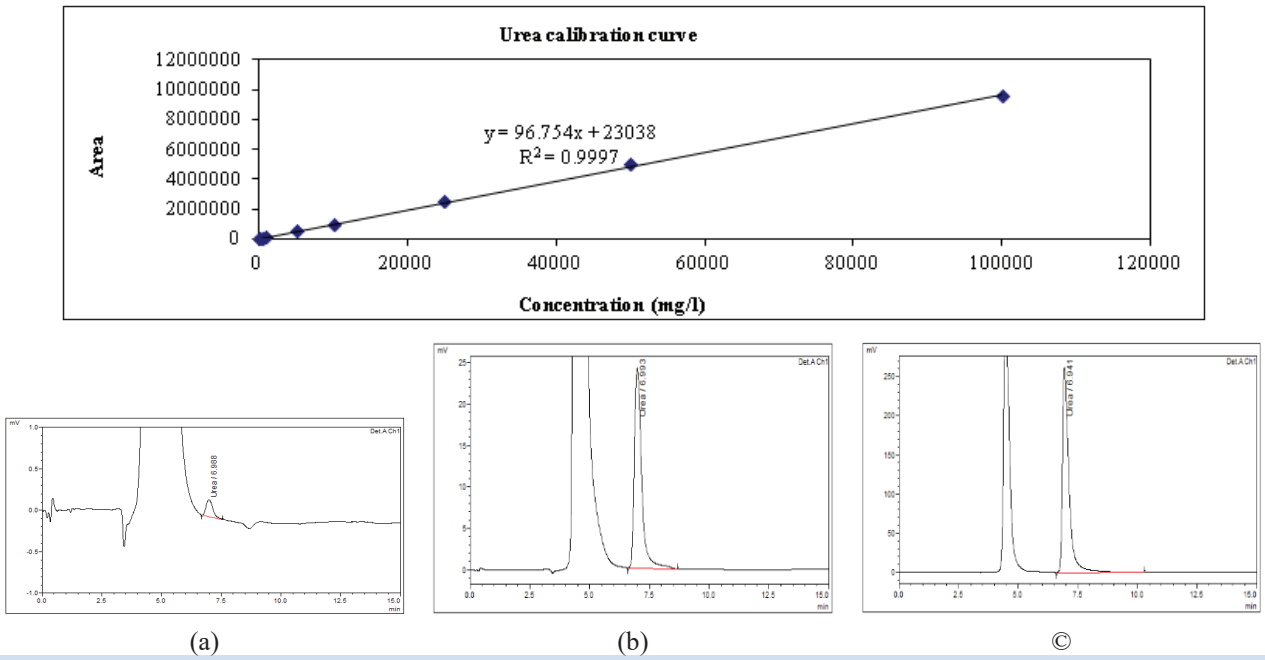
The method validation results of urea analysis by in-house HPLC-RID method were given in Table 3. Calibration curve of urea was created within 25, 50, 100, 500, 1000, 5000, 10000, 25000, 50000 and 100000 mg/l concentrations. y=96.754x+23038 linear regression equations with R^2:0.9997 coefficient of determination was found, indicating that a good linearity was achieved. Retention times of urea were between 6.94-6.99 minutes (Figure 2). In the developed method, the limit of detection (LOD) value for urea was determined as 75 mg/kg and the limit of quantification (LOQ) value was determined as 250 mg/kg.

For the precision of the method, repeatability and reproducibility were determined as 1.08% and 1.84%, respectively (Table 3).

For accuracy, the recovery was calculated from repeatability and reproducibility studies and determined as 95.10%. Obtaining high recovery showed that this developed method had suitable sensitivity for the determination of urea in different concentrations in DCF.

Moisture and Urea Content of DCF

The moisture and urea contents of DCF determined in blank samples by HPLC-RID, SC-420 and SC-435 methods were given in Table 4. The urea content of DCF 1 used in optimization and validation of urea analysis by in-house HPLC-RID method was determined as 'Not Detected' by all three methods. The amount of urea in the UF-DCF group by in-house



(a) (b) ©

Figure 2. Urea calibration curve and urea standard chromatograms in the measurement range of 25-100000 mg/l

a: Chromatogram of the 50 mg/l urea standard, b: Chromatogram of the 5000 mg/l urea standard, c: Chromatogram of the 50000 mg/l urea standard

Table 3. In-house HPLC-RID validation results

Parameters	Data
Linear range 25-100000 (mg/kg)	R^2=0.9997
Limit of detection (LOD) (mg/kg)	75.00
Limit of quantification (LOQ) (mg/kg)	250.00
Repeatability (%)	1.08
Reproducibility (%)	1.84
Recovery (%)	95.10

Table 4. The moisture and urea contents of dairy cattle feed determined in blank samples by HPLC-RID, SC-420 and SC-435 methods

Feed materials	Feed urea level	Moisture (%)	Urea content (%)*		
			HPLC-RID	SC-420	SC-435
Method optimization and validation feed material					
DCF 1	Urea-free	11.81	N.D	N.D	N.D
Trial groups feed materials					
DCF 2	Urea-free	16.25	N.D.	0.21	0.12
DCF 3		9.15	N.D.	0.21	N.D.
DCF 4		9.95	N.D.	0.37	0.29
Mean±SEM			N.D.^{c}	0.26±0.053^{a}	0.16±0.066^{b}
DCF 5	Low urea	10.40	0.16	0.15	0.13
DCF 6		14.95	0.17	0.24	0.21
DCF 7		13.01	0.18	0.32	0.25
DCF 8		8.92	0.17	0.28	0.26
Mean±SEM			0.17 ± 0.004^{b}	0.25±0.034^{a}	0.19±0.049^{b}
DCF 9	High urea	12.29	1.38	1.05	1.06
DCF 10		10.02	1.39	1.40	1.42
DCF 11		10.15	1.51	1.60	1.62
Mean±SEM			1.43 ± 0.533^{a}	1.35±0.161^{b}	1.37±0.164^{b}

*corrected according to 12% humidity.

a, b: Within a line, means followed by different letter differ significantly (P<0.05).

N.D: Not detected (HPLC-RID LOQ: 250 mg/kg<, SC-420 and SC-435 LOQ: 0.12%<), DCF: dairy cattle feed, HPLC-RID: Refractive index detector HPLC, SC-420 nm: Spectro-Colorimetry 420 nm, SC-435: Spectro-Colorimetry 435 nm.

HPLC-RID method was determined as 'Not Detected' while it was determined as 0.26±0.053% in SC-420 and 0.16±0.066% in SC-435 (P<0.05). In the LU-DCF group, similar urea amounts were determined by the in-house HPLC-RID method (0.17 ± 0.004%) and the SC-435 method (0.19±0.049%) (P>0.05), while higher results were obtained by the SC-420 method (0.25±0.034%) (P<0.05). In the HU-DCF group, urea amounts were detected close to each other with SC-420 and SC-435 (P>0.05), and higher by in-house HPLC-RID method (P<0.05).

Urea Content of the Trial Groups and Recoveries

The urea content and recoveries determined by the HPLC-RID, SC-420 and SC-435 methods of the trial groups were given in Table 5. The effects of the methods used in this study on the urea content and recoveries of the trial groups were found to be significant and higher values by the HPLC-RID method were found as compared to that of spectro-colorimetric method (P<0.01). The effect of the urea level of UF-DCF, LU-DCF and HU-DCF were insignificant to the recovery and urea concentrations found in HPLC-RID (P>0.01). Similar results of urea content and recovery were found with all three methods in LU-DCF, HU-DCF and UF-DCF within the range of 25-100000 mg/kg (P>0.01).

The interaction effect of method x urea level in feed on the urea content and recoveries of the trial groups is significant (P<0.01). The highest urea content and recoveries were determined in LU-DCF, HU-DCF and UF-DCF respectively by SC-420 and SC-435 methods, and in UF-DCF, HU-DCF and LU-DCF respectively by HPLC-RID method (Figure 3).

DISCUSSION

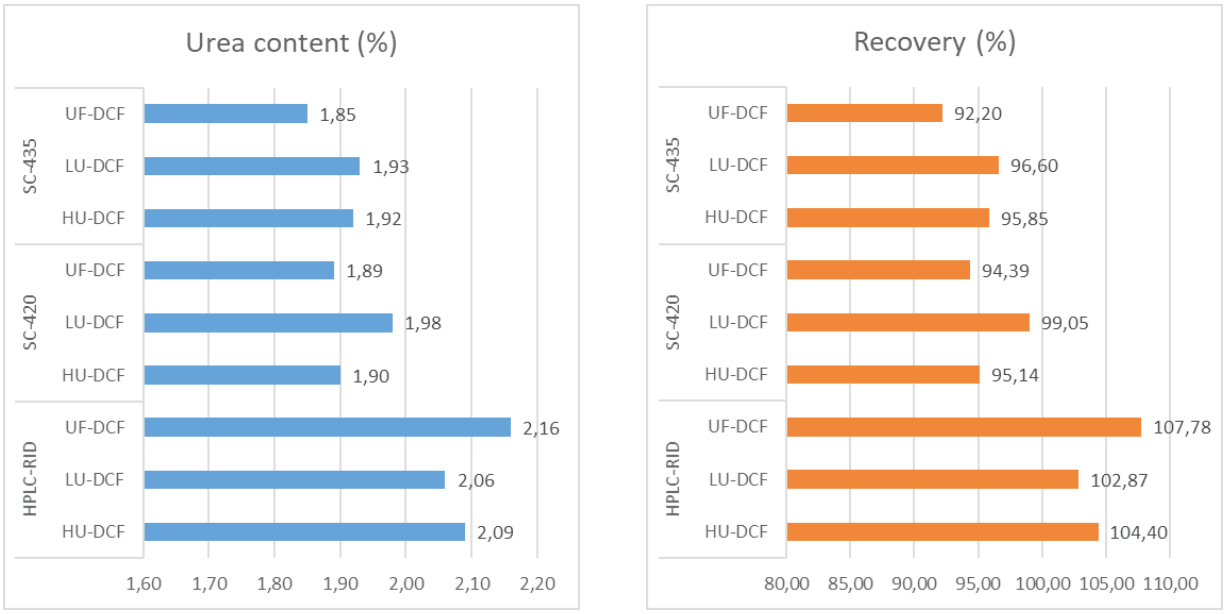
In this study, the aim was to develop a sensitive, reliable, and accurate method for analyzing the urea content of DCF with HPLC-RID. Four different methods were reported for the determination of urea by HPLC in feed materials (Pibarot and Pilard, 2012; Wegh et al., 2018; Flannelly et al., 2019; Krämer et al., 2021). However, the method for the determination of urea in DCF with HPLC-RID has not yet been reported.

In pet food, it was reported that the filtrates of spectro-colorimetric method were reacted with 4-DMAB, and obtained a mixture. The mixture was analyzed in LC/UV-DAD, leading that the detection of urea was enabled in LC/UV-DAD (Pibarot and Pilard, 2012). In animal feed, ultra-high performance liquid chromatography (UHPLC) method depending on the atmospheric-pressure chemical ionization (APCI) and

Table 5. Urea content and recoveries of trial groups determined by HPLC-RID, SC-420 and SC-435 methods.

		N	Spiking concentration (%)	Urea content (%)	P-value	Coefficient of variation (%)	Recovery (%)	P-value
Method								
HPLC-RID		50	2.0	2.10±0.013^{a}	0.0001*	5.43	105.02±0.64^{a}	0.0001*
SC-420		50	2.0	1.93±0.013^{b}		3.97	96.19±0.64^{b}	
SC-435		50	2.0	1.90±0.013^{b}		4.98	94.96±0.64^{b}	
Feed urea level								
LU-DCF		60	2.0	1.99±0.011	0.2643	5.48	99.51±0.56	0.2779
HU-DCF		45	2.0	1.97±0.013		5.52	98.46±0.66	
UF-DCF		45	2.0	1.99±0.013		8.48	98.20±0.66	
Method x Feed urea level								
HPLC-RID	LU-DCF	20	2.0	2.06±0.020^{ab}	0.0001*	4.64	102.87±0.997^{ab}	0.0001*
	UF-DCF	15	2.0	2.16±0.023^{a}		6.09	107.78±1.152^{a}	
	HU-DCF	15	2.0	2.09±0.023^{ab}		4.74	104.40±1.152^{ab}	
SC-420	LU-DCF	20	2.0	1.98±0.020^{bc}		4.08	99.05±997^{bc}	
	UF-DCF	15	2.0	1.89±0.023^{cd}		2.90	94.39±1.152^{cd}	
	HU-DCF	15	2.0	1.90±0.023^{cd}		2.48	95.14±1.152^{cd}	
SC-435	LU-DCF	20	2.0	1.93±0.020^{cd}		5.87	96.60±0.997^{cd}	
	UF-DCF	15	2.0	1.85±0.023^{d}		4.53	92.42±1.152^{d}	
	HU-DCF	15	2.0	1.92±0.023^{cd}		2.49	95.85±1.152^{cd}	

*Within a column, means followed by different letter differ significantly (P<0.05).
HPLC-RID: Refractive index detector HPLC, SC-420 nm: Spectro-Colorimetry 420 nm, SC-435: Spectro-Colorimetry 435 nm., LU-DCF: low urea dairy cattle feed, HU-DCF: high urea dairy cattle feed, UF-DCF: urea-free dairy cattle feed



(a) (b)

Figure 3. The interaction effect of urea amount and recovery method x feed urea level.

a: method x feed urea level interaction effect on urea amount
b: method x feed urea level interaction effect on recovery

tandem mass spectrometry (MS/MS) was developed to analyze urea content (Wegh et al., 2018). In yeast based foods and feed-grade yeast to determine urea content via spectro-colorimetric methods, enzymatic methods and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis, 4 different laboratories` results for urea content were compared. It was reported that the urea determined by spectro-colorimetric method in-laboratory and inter-laboratory results were not consistent, and the most consistent and reliable results were obtained by using the LC-MS/MS method (Flannelly et al., 2019). In compound feed, pet food and yeasts, two methods were defined for determining urea after derivatization with xanthydrol in liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and high-performance liquid chromatography fluorescence detector (HPLC-FID) (Krämer et al., 2021).

The LC-MS/MS can be used for sensitive, accurate and reliable detection of urea in feed materials (Pibarot and Pilard, 2012; Wegh et al., 2018; Flannelly et al., 2019; Krämer et al., 2021). This advanced and expensive technology requires a high financial investment and is therefore difficult to find in every laboratory. In contrast, the HPLC-RID is cheaper than the LC-MS/MS.

Method Development and Optimization

A number of preliminary studies were conducted for method development. Conditions such as urea sensitive column and mobile phase selection, column oven temperature, flow rate, sample extraction, analysis time were evaluated as analytical studies for method development. In this study, the results of study involving only optimization of mobile phase and sample extraction were shared.

Regarding the mobile phase conditions, the use of acetonitrile/water combinations instead of preparing buffer solutions in determining urea with HPLC-RID was an advantage. The use of acetonitrile/water combinations as a mobile phase offered a faster and simpler preparation possibility. In addition, it reduced the tendency of precipitation and clogging within the chromatographic system. It was determined that the retention time delayed as the proportion of acetonitrile/water in the mobile phase increased (Table 1, Figure 1). In mobile phase optimization, the concentrations obtained according to urea standards in the (85/15) and (90/10) acetonitrile/water (v/v) mobile phases were obtained as 1.85% and 2.06% respectively, and with a 2% urea spike to UF-DCF, close results were obtained as 1.99% and 2.08%. However, in order for the separation of urea peak from mobile phase peak to be clear, (90/10) acetonitrile/water (v/v) mobile phase was preferred in studies to be conducted with real samples. For real samples, a peak belong-

ing to another analyte is obtained between the mobile phase and the urea peak, and in this case distinguish the urea peak may not be possible.

50 ml volumetric flasks were used. It is known that water is used as a solvent in the spectro-colorimetric method for the extraction of urea from the sample. In addition, water was also used as a solvent in the studies conducted on the determination of urea by the HPLC method in animal feed (Pibarot and Pilard, 2012; Wegh et al., 2018; Flannelly et al., 2019; Krämer et al., 2021). Acetonitrile was used in the extraction of urea in this study. The use of acetonitrile in the extraction of many analytes for the protein precipitation process was reported by many researchers (Ashri and Abdel-Rehim, 2011; Galecio et al., 2022). In addition, the use of acetonitrile is one of the fastest methods for the process of precipitation of proteins from biological samples (Ashri and Abdel- Rehim, 2011).

In order to optimize the sample extraction, different sample amount and shaking times were applied. Thus, the extraction process, which can be obtained correctly and in a short time, was defined. The most suitable urea in this study and the recovery results were obtained with a sample amount of 2.5 g and 5.0 g. A sample amount of 5.0 g was used in the analysis of the real samples after optimization. Because if the extraction is performed in a 50 ml volumetric flask, the dilution coefficient is small. This gave us the advantage of reducing analytical errors and process practicality. The shaking time, sample amount x shaking time interactions statistically effected recovery rate insignificantly ($P>0.01$), indicating that urea extraction can be completed within 10 minutes shaking time in the method developed in the study. Taken together, a 10-minute shaking time was used for the extraction of real samples.

Method Validation

The measurement range of 25-100000 mg/kg obtained in the study was higher than 0.01- 10 mg/kg determined in various fermented foods and beverages by Matsudo and Sasaki (1995), 10-2000 mg/kg determined in milk by Dai et al. (2012) with liquid chromatography-isotope dilution mass spectrometry (HPLC-IDMS), 0.1-500 mg/l determined by HPLC-FLD combined with derivatization of urea before colon in canned foods by Zeng et al. (2015). R^2:0.9997 linearity was similar to the values obtained by Dai et al. (2012) as r^2: 0.9995, Wang et al. (2014) as r^2:0.9993 and Zeng et al. (2015) as r^2:0.9995. Therefore, the method developed in this study allowed the determination of urea in DCF through a wide range with good linearity.

The values of 75 mg/kg LOD and 250 mg/kg LOQ determined in this study were higher than some other research findings (Matsudo and Sasaki, 1995; Pibarot and Pilard, 2012; Wang et al., 2014; Zeng et al., 2015; Flannelly et al., 2019; Krämer et al., 2021). The fact that the LOD and LOQ values of the developed method are high is due to the fact that the Refractive Index Detector is has the lowest sensitivity among other HPLC detectors (Akkoç, 2023). The LOD and LOQ values have appropriate and sufficient sensitivity for determining 0.3-0.5 g/kg/day (between 120 and 200 g for 400 kg cattle) toxic and 1.0-1.5 g/kg/day (between 400 and 600 g for 400 kg cattle) lethal urea doses for cattle (Anonymous, 2023).

1.08% for the repeatability RSD and 1.84% for reproducibility RSD found in this study were higher than; repeatability in milk (0.15-0.46%) and repeatability in milk powder (0.18-0.65%), which were conducted in isotope dilution gas chromatography-mass spectrometry (GC/IDMS) (Dai et al., 2010). Moreover, repeatability in milk (0.17-0.38%) and reproducibility in milk (0.28-0.40%) reported by Dai et al. (2012) were lower than that of this study. Results obtained in this study were found to be compatible with 1.4-7.2% variation coefficient in various food and feeds, in which HPLC-FLD with repeatability RSD 1.9-2.3% (Wang et al., 2014) and repeatability RSD 2.05-6.53%, (Zeng et al., 2015) and repeatability RSD 1.4-4.7% (Krämer et al., 2021) was reported.

The 95.10% recovery value obtained in this study was lower than that of Matsudo and Sasaki (1995) at 100 ppm spike concentration, that of Dai et al. (2012) by spiking urea into 4 milk samples. The recovery value we found was compatible with 80.2-109.7% (Zeng et al., 2015), 86-105% (Krämer et al., 2021). The validation results of the study show that this method was efficient, highly recoverable and repeatable.

The Amount of Urea and Recovery Rates Determined by HPLC-RID, SC-420 and SC-435 Methods

The amount of urea in blank samples of UF-DCF was determined as 'Not Detected' by the HPLC-RID method, while the positive presence of urea was determined as 0.26±0.053% with SC-420 and 0.16±0.066%

with SC-435. For blank samples of LU-DCF, HPLC-RID and SC-435 methods` results were close to each other as 0.17 ± 0.004% and 0.19±0.049% urea content respectively. On the contrary SC-420 methods showed 0.25±0.034% urea content, which was higher than two other methods used in the study. These results show that the spectro-colorimetric method is to determining trace levels of urea, and it cannot be used for precise detection of the exact presence or absence of urea. These contradictory results were also reported by many studies (Pibarot and Pilard, 2012; Flannelly et al., 2019; Krämer et al., 2021). In addition, the determination of lower urea amounts at 435 nm wavelength compared to the measurement at 420 nm wavelength in the spectro-colorimetric method proves that some of the amino acids in DCF were eliminated (Pibarot and Pilard, 2012).

In blank samples of HU-DCF, urea contents were found to be similar to each other with SC-420 and SC-435 ($P>0.05$), and higher by in-house HPLC-RID method ($P<0.05$). The determination of the lower amount of urea in the spectro-colorimetric method (420 nm wavelength and 435 nm wavelength) compared to the HPLC-RID urea method may be due to the fact that the reaction of 4-DMAB with the urea molecule in the filtrates of HU-DCF does not occur with sufficient efficiency. Obtaining higher results with the in-house HPLC-RID method proved that the HPLC method is more reliable. The reason for the high urea results in the developed method is the use of low-volume volumetric flasks for sample extraction. Obtaining low urea results in spectro-colorimetric methods suggests that there are losses. Because it is reported by many authors that there are problems in the derivatization stage of filtrate with 4-DMAB in the spectro-colorimetric method (Giraldo and Rivas, 2017; Hussain et al., 2022). Molar concentration of DMAB and acid, differences in solvent type change the reaction efficiency and cause unrepeatable results (Giraldo and Rivas, 2017; Hussain et al., 2022).

The effect of the method on the urea content and recoveries of the trial groups was found to be significant, and higher values were determined by the HPLC-RID method. In addition, similar results were found with all three methods in terms of urea content and recovery rates of LU-DCF, HU-DCF and UF-DCF ($P>0.01$). It was proven that this method can be used instead of the spectro-colorimetric method, which is the official standard method. These results show that the precise result can be obtained above the 2% level of urea, which is the legal limit for urea in Türkiye.

The interaction effect of method x feed urea level on the urea content and recoveries of the trial groups was found to be significant ($P<0.01$). The highest amount of urea and recoveries were obtained by in-house HPLC-RID method at all feed urea levels. This result reveals the superiority of the developed method compared to the spectro-colorimetric method. The reason for the significance of the interaction effect may be due to inconsistent results obtained in the spectro-colorimetric method. Because the DCF that make up the trial groups were supplied from the market, the composition of the raw materials and the feed additives used are unknown. The fact that compound feed contains soy product, the possibility of using synthetic methionine and feed yeast as feed additives may have an effect on the results obtained in the study. Compound feed is a complex material for the determination of urea analyte. Feed raw materials or feed additives in compound feed may have had an adverse interaction with urea. Soy products of Leguminosae origin in animal feed can also show urease activity after compound feed production (Follmer, 2008). Urease activity in feed may cause the hydrolyzation of urea, resulting in low urea concentration. During the extraction of urea from feed, feed yeast is also able to hydrolyze urea by having urease activity (Flannelly et al., 2019). The opposite is the case with methionine as a feed additive. Methionine might react with 4-DMAB, causing high urea concentrations in compound feed. As a matter of fact, Pibarot and Pilard (2012) performed urea analysis by spectro-colorimetric method using 2 different batches of methionine in their study and found the presence of urea at 5.56% and 4.67% levels, respectively. When they performed urea analysis by enzymatic method using the same amino acids, they did not determine the presence of urea. The researchers reported that methionine was determined like urea by spectro-colorimetric method.

CONCLUSION

It was concluded in this study that the in-house HPLC-RID urea method developed can be used on real samples in the measurement range of 25-100000 mg/kg in DCF. It was shown that this method developed is a more sensitive, fast, and reliable method compared to the spectro-colorimetric method. It is also thought that it can be used for the determination of urea with raw materials similar to DCF ration formulations like beef cattle feed, sheep feed, goat feed

ruminant feeds, etc. In institutions providing laboratory services, it is recommended to perform method validation before the in-house HPLC-RID urea method is put into use. In addition, it may be recommended to use in routine controls of the in-house HPLC-RID method. For verification, LC-MS/MS method developed by other researchers can be used. The developed in-house HPLC-RID urea method has the potential to become a standard method for determining urea in feed.

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ETHICAL APPROVAL

The study was approved by the editorial boards of the authors' institutions.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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