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Research Article

Development of an LC-MS/MS method for the determination of difucosyllactose in infant formula

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Abstract

Difucosyllactose (DFL) is a type of human milk oligosaccharide (HMOs) that is added to infant formula to support gut health, immune development, and the establishment of a beneficial gut microbiota in infants. In this study, LC-MS/MS techniques were used to determine the DFL content in infant formula. The powdered milk samples were reconstituted with water, incubated with 10 U/mL of amyloglucosidase enzyme for 30 min, and then the protein was precipitated with 50% acetonitrile. A Waters ACQUITY UPLC BEH Amide chromatographic column (150 mm × 2.1 mm, 1.7 µm) was used and maintained at 55°C during analysis, with the mobile phase consisting of 10 mM ammonium formate and acetonitrile (ACN) in a gradient program. The samples were detected using a mass spectrometer with negative electrospray ionization mode and multi-reaction monitoring (MRM) using the ion transition m/z 633.5 → 325.58 and m/z 633.5 → 247.2 for quantification and qualification, respectively. The method was validated according to AOAC and SMPR® 2021.006 guidelines. The results showed good specificity, with a linear range of 1 - 20 µg/mL. The detection and quantification limits of the method were 4.80 - 5.12 µg/g and 11.2 - 17.1 µg/g, respectively. The method was then applied to analyze the DFL content in 24 samples of commercial infant formula purchased in Hanoi. The results showed that the DFL content in the samples ranged from 15.6 - 214 mg/100 g, with significant differences observed between age groups and product types.

Keywords: Difucosyllactose, DFL, Lactodifucotetraose, LDFT, Human Milk Oligosaccharides, HMOs, LC-MS/MS.

1. INTRODUCTION

HMOs (Human Milk Oligosaccharides) are the third most abundant solid component in breast milk after fats and carbohydrates and play a vital role in the growth and development of infants and young children [1, 2]. Due to their structural diversity, more than 200 substances have now been discovered, with several already studied. Although present in lower amounts in breast milk than other HMOs, averaging from 0.3 - 0.5 g/L [3] depending on lactation stage, Difucosyllactose (DFL) plays a role in modulating the infant gut microbiome. It acts as a prebiotic to support the growth of beneficial bacteria, particularly *Bifidobacterium* species [1, 4], and is currently being further studied and added to products [5].

Currently, several standards regulate the addition of DFL to infant formula, typically as a mixture with 2'-fucosyllactose (2'-FL) containing a minimum DFL content of 5% on a dry basis. The maximum permissible levels of the 2'-FL/DFL mixture vary by jurisdiction and age group. For instance, the EU limits the

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concentration to 1.2 g/L in the final product ready for use according to the manufacturer's instructions in infant formula [6, 7], while FSANZ sets a limit of 96 mg/100 kJ [8]. Meanwhile, the FDA permits the use of 2'-FL/DFL in non-exempt formulas for term infants at a maximum of 1.6 g/L; in toddler drinks at 1.2 g/L; in other infant foods at 10 g/kg; and in conventional dairy products for the general population at levels ranging from 2 to 40 g/kg [3].

Quantifying DFL is essential for controlling product quality and meeting international export standards. However, few laboratories currently offer DFL testing services, and published research on DFL quantification remains limited. Therefore, this study aims to develop and validate a method for quantifying DFL in milk-based supplements and implement it at the National Institute for Food Control. This research will provide valuable technical data for the determination of DFL in food matrices.

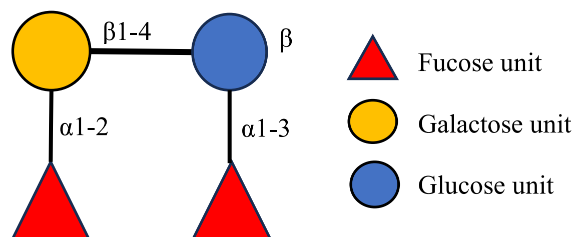

Figure 1. *Chemical structure of difucosyllactose*

Difucosyllactose is also known as Lactodifucotetraose (LDFT), molecular formula $C_{24}H_{42}O_{19}$, molecular weight of 634.55. Its structure consists of a lactose unit (Gal-β1-4-Glc) linked to two fucose groups via α-1-2 and α-1-3 glycoside bonds as follows: α-L-Fuc-(1→2)-β-D-Gal-(1→4)-[α-L-Fuc-(1→3)]-β-D-Glc (**Figure 1**) [9, 10]. Due to structural similarity of DFL to other HMOs and the high levels of lactose, protein, and other components that can interfere with its detection, the determination of DFL in infant formula presented analytical challenges. While liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) offers high sensitivity and selectivity, background effects, particularly ion suppression, can negatively impact the accuracy and reproducibility of quantification [11]. To address these challenges, in this study, amyloglucosidase enzyme (AMG) was used to hydrolyze carbohydrates, resulting in improved recovery and accuracy of DFL determination [12].

Amyloglucosidase plays a crucial role in the analysis of HMOs by eliminating interfering polysaccharides such as starch and glycogen, from food matrices. During hydrolysis, AMG cleaves α-1,4 and α-1,6 glycosidic bonds, converting long-chain polysaccharides into glucose. This enzymatic clean-up step effectively reduces matrix interference prior to LC-MS/MS analysis [13-15]. Consequently, this study optimized the sample preparation conditions using AMG, followed by LC-MS/MS quantification. The method was subsequently validated for accuracy and precision to ensure its analytical reliability.

2. MATERIALS AND METHODS

2.1. Instruments and chemical

2.1.1. Instruments and equipment

The study was conducted using a high-performance liquid chromatography system coupled with tandem mass spectrometry (LC-MS/MS), specifically an H-Class system equipped with a Xevo TQD detector (Waters, USA). Other laboratory equipment included an Elma ultrasonic bath, a Hermle Z326K centrifuge and a GFL temperature-controlled water bath, all of which were utilized during the analytical procedures.

2.1.2. Samples

For method validation, three matrix blanks with no HMOs present were analyzed by LC-MS/MS. These included one powdered infant formula product, one ready-to-feed milk product, and one probiotic yogurt product. The selected matrix blanks were used to represent the three major infant formula product categories (powdered infant formula, ready-to-feed milk, and probiotic yogurt) and used for the evaluation of method specificity, accuracy, precision and matrix effects.

Following method validation, 24 commercial products declaring the presence of human milk oligosaccharides (HMOs), including difucosyllactose (DFL), were purchased from retail stores in Hanoi,

Vietnam. The samples comprised 14 powdered infant formulas, 7 ready-to-feed milk products, and 3 probiotic yogurt drinks, and were analyzed using the validated LC-MS/MS method.

2.1.3. Chemical

All chemicals and reagents used in this study were of mass spectrometry-grade purity. Difucosyllactose (DFL, purity ≥ 90.0%, batch No. PA0092-RA01) was purchased from Elicityl (France). Deionized water was produced using a Milli-Q water purification system. Methanol, acetonitrile, and glacial acetic acid were obtained from Merck (Germany). Ammonium formate and sodium hydroxide were supplied by Fisher Scientific (USA).

Amyloglucosidase powder from *Aspergillus niger*, purchased from Megazyme (Ireland), had activity about 3,260 U/mL at 40°C, pH 4.5 on soluble starch). The AMG working solutions were prepared by diluting the enzyme powder in 0.1 M sodium acetate buffer at pH 4.5 to obtain concentrations within the research range of 6, 8, 10, 12, and 14 U/mL. All solutions were prepared fresh and used on the same day.

A difucosyllactose (DFL) stock standard solution at a concentration of 1000 µg/mL was prepared in water and stored at -20 ± 4°C. Calibration standard solutions in the concentration range of 1 - 20 µg/mL were freshly prepared daily.

2.2. Method

2.2.1. Sample preparation

Based on a review of the relevant literature [9, 11, 17, 18] the proposed sample preparation procedure was as follows: powdered milk samples (5.0 g) were accurately weighed and completely dissolved to 50 mL of water in a volumetric flask. An aliquot of 200 µL of the reconstituted sample was transferred into a 2 mL centrifuge tube, followed by the addition of 300 µL of amyloglucosidase enzyme solution and the mixture was incubated at 65°C. Proteins and lipids were precipitated using acetonitrile at an optimized concentration determined during method development. The sample was then centrifuged at 12,000 rpm for 5 min and the resulting supernatant was filtered through a 0.22 µm cellulose acetate membrane filter prior to LC-MS/MS analysis.

Sample preparation was optimized using a univariate approach on a blank matrix (prepared according to Section 2.1.2) and spiked with the analyte at 16 mg/100 g. Each parameter was evaluated by varying a single factor while maintaining all other variables constant. Working enzyme solutions were prepared in a 0.1 M sodium acetate buffer solution pH 4.5, as described in Section 2.1.3. While the incubation was fixed at 65°C, the following variables were examined, enzyme concentration (6 - 14 U/mL), incubation time (10 - 50 min) and acetonitrile concentration for protein precipitation (30 - 70%, v/v).

Each experiment was performed in triplicate, with data expressed as the mean ± standard deviation (SD).

2.2.2. Methods of analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was employed for the analysis of DFL in infant formula. The mass spectrometry parameters were optimized based on existing literature and preliminary laboratory experiments.

2.2.3. Validation of method

Method validation was conducted in accordance with the AOAC Appendix F: Guidelines for Standard Method Performance Requirements [19] and the AOAC SMPR® 2021.006 Standard Method Performance Requirements (SMPRs®) for the determination of difucosyllactose (DFL) [20] in infant and adult/pediatric nutritional formulas. The validation parameters included system suitability, specificity, calibration curve, method detection limit (MDL), method quantification limit (MQL). Accuracy was determined via recovery studies, while precision was assessed through repeatability and expressed as relative standard deviation (RSD%) across three concentration levels.

2.2.4. Data processing

Data acquisition and processing were performed using MassLynx software (Waters Corp.), while statistical analysis was conducted using Microsoft Excel 2019.

3. RESULTS AND DISCUSSION

3.1. LC-MS/MS parameters

Based on literature reviews and preliminary experiments [11], a Waters ACQUITY Amide column (150 × 2.1 mm, 1.7 µm) was selected to optimize chromatographic separation conditions. The flow rate was set at 0.4 mL/min and the column temperature was maintained at 55°C. The mobile phase, which was examined for its ability to separate DFL from 6 common HMOs including 2'-Fucosyllactose, 3-Fucosyllactose, 3'-Sialyllactose, 6'-Sialyllactose, Lacto-N-tetraose, and Lactose-N-neotetraose (data not shown), consisted of solvent A (10 mM ammonium formate) and solvent B (acetonitrile). The gradient program was as follows: 0 - 5 min, 24% to 26% A; 5 - 7 min, held at 26% A; 7 - 8.5 min, 26% to 60% A; 8.5 - 10.5 min, 60% A; 10.5 - 10.7 min, 60% to 24% A; and 10.7 - 14 min, 24% A for column re-equilibration.

Mass spectrometry was performed on a Waters Xevo TQD system operating in negative electrospray ionization (ESI (-)) mode using multiple reaction monitoring (MRM). MS parameters were automatically optimized via MassLynx software. The most intense MRM transition was selected for quantification, while the second most intense was used for qualitative confirmation. The source conditions were established as follows: capillary voltage, 2.5 kV; desolvation temperature, 500°C; and nitrogen (N_2) desolvation gas flow, 850 L/h. Argon served as the collision gas. DFL was monitored using the precursor-to-product ion transitions of 633.5 → 325.58 (quantifier) and 633.5 → 247.2 (qualifier), with respective collision energies of 10 and 20 eV (**Figure 2**).

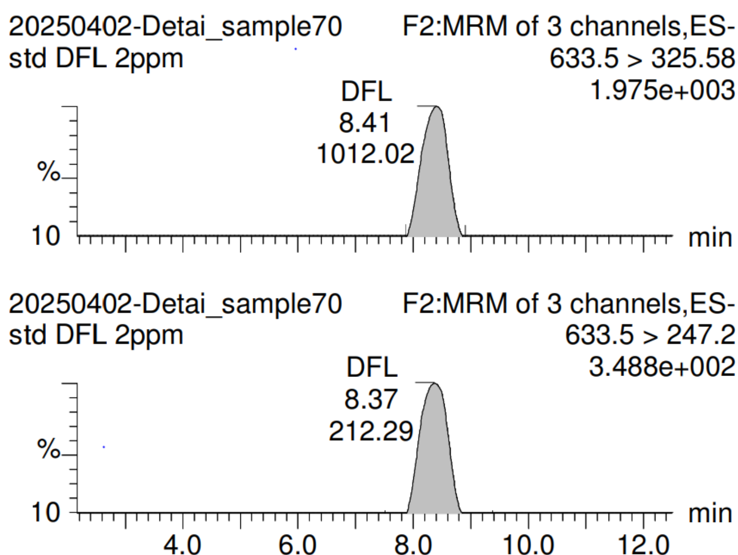

***Figure 2**. Representative LC-MS/MS chromatogram of DFL under selected conditions*

Based on structural predictions, the formation of the fragment at *m/z* 325 may result from the cleavage of the glycosidic bond between glucose and galactose, yielding a disaccharide $[Fuc+Gal+OH]^-$ fragment. This fragment also serves as the product ion for identifying 2'-Fucosyllactose [10, 11, 17] and may be considered a characteristic fragment of the fucosyllactose group. Subsequently, cross-ring cleavage of the reducing-end glucose to form the ion $[Fuc+C_4H_6O_4]^-$ at *m/z* 247 [21]. By monitoring one precursor ion and two products ion via LC-MS/MS, the method achieved 5 identification points (IP), fulfilling the specificity requirements [22] for DFL analysis. Nevertheless, to fully elucidate the relationship between the molecular structure of DFL and its fragmentation patterns, further specific studies are required.

3.2. Sample preparation optimization

3.2.1. Enzyme concentration

Transferred 200 µL of the reconstituted sample as in Section 2.2.2, added 300 µL of AMG working solution. Enzyme concentrations were examined at five levels: 6, 8, 10, 12 and 14 U/mL. The mixture was incubated at 65°C for 30 min. The results are presented in **Figure 3**.

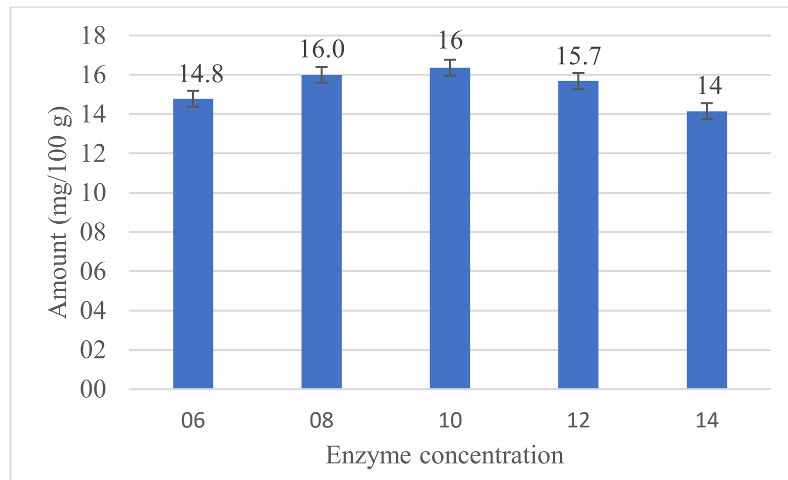

Figure 3. *Results on different enzyme concentrations*

As illustrated in **Figure 3** show that increasing the AMG concentration from 6 to 10 U/mL resulted in a significant elevation in DFL recovery, from 14.8 ± 0.51 to 16.4 ± 0.28 mg/100 g ($p < 0.05$). This trend suggests that higher enzyme concentrations loading facilitates the enzymatic hydrolysis of interfering polysaccharides into monosaccharides, thereby eliminating co-extracted interference and mitigating the matrix effect. However, a further increase to 12 and 14 U/mL resulted in a gradual decline in DFL content to 15.7 ± 0.25 and 14.1 ± 0.34 mg/100 g, respectively. This decrease may be attributed to the side-reactions catalyzed by traces glucosidases impurities, potentially leading to the formation of byproducts that interfere with determination of DFL [13-15].

3.2.2. Enzymatic incubation time

A 200 µL aliquot of the reconstituted sample (prepared as described in Section 2.2.2) was mixed with 300 µL of a 10 U/mL AMG working solution. The mixture was subsequently incubated at 65°C for various durations (10 - 50 min). The influence of incubation time on the analytical response is shown in **Figure 4**.

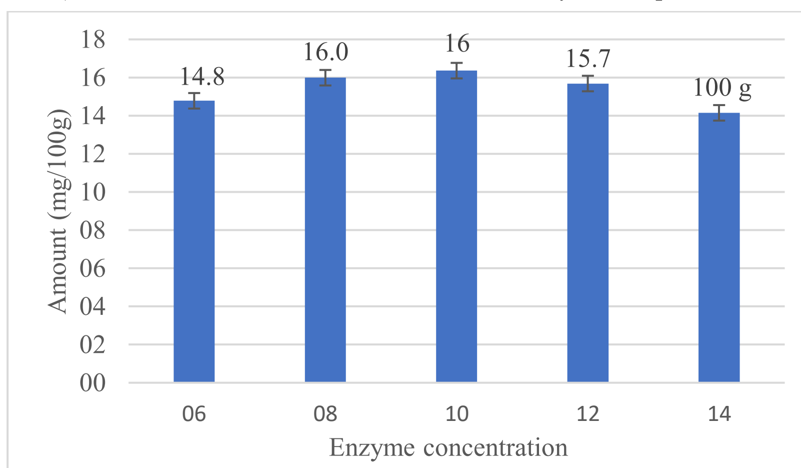

Figure 4. *Results on enzyme incubation durations*

As illustrated in **Figure 4**, increasing the incubation time from 10 to 30 min significantly enhanced DFL recovery, indicating more effectively removal of interfering polysaccharides. No statistically significant difference was observed between 30 and 40 min, suggesting a plateau in the enzymatic reaction. However, a slight decrease in DFL content was noted at 50 min, which may be attributed to thermal degradation [21]. Therefore, an incubation time of 30 min was selected as the optimal duration to balance analytical efficiency with maximum DFL recovery.

3.2.3. Precipitation solvent

Following enzymatic incubation, the acetonitrile (ACN) proportion in the sample solution was varied from 30% to 70% (v/v) to evaluate its effect on protein precipitation and DFL recovery. The influence of ACN concentration on the extraction efficiency is presented in **Figure 5**.

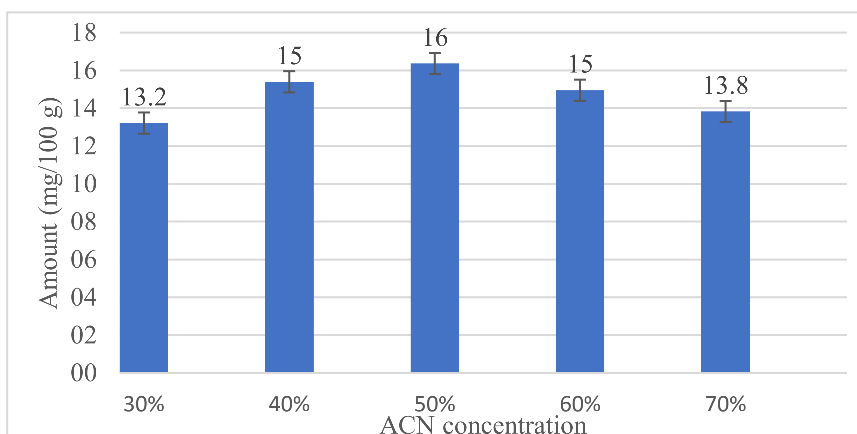

Figure 5. *Results of precipitation solvent*

As illustrated in **Figure 5** show that, increasing the ACN content from 30% to 50% resulted in a significant increase in DFL recovery, from 13.2 ± 0.046 to 16.4 ± 0.28 mg/100 g. This trend indicates that higher ACN levels effectively facilitate protein precipitation and the removal of matrix interferences. Conversely, further increasing the ACN proportion to 60% and 70% resulted in a substantial decline in DFL recovery. This reduction may be attributed to the co-precipitation of DFL or its decreased solubility in high-organic solvent environments. Consequently, 50% ACN was selected as the optimal concentration for protein precipitation, a finding that aligns with the known solubility characteristics of Human Milk Oligosaccharides (HMOs) [23].

3.2.4. Selected procedure

Based on the optimization of sample preparation, the finalized procedure was established as follows: Powdered milk samples (~5.0 g) were accurately weighed and dissolved to 50 mL of deionized water in a volumetric flask, while ready-to-feed milk and probiotic yogurt samples were appropriately diluted. A 200 µL aliquot of the sample solution was transferred to a 2 mL centrifuge tube and mixed with 300 µL of 10 U/mL amyloglucosidase. The mixture was incubated at 65°C for 30 min. Subsequently, 500 µL of 50% (v/v) acetonitrile was added to facilitate the precipitation of proteins and lipids. Following centrifugation at 12,000 rpm for 5 min, the resulting supernatant was filtered through a 0.22 µm cellulose acetate membrane prior to LC-MS/MS analysis.

The DFL content (mg/100 g) in the sample is calculated using the formula:

$$X = \frac{\mathrm{C} \times \mathrm{V} \times \mathrm{k} \times 5}{m \times 10}$$

Where: C is the DFL concentration calculated from the calibration curve (µg/mL), V is final volume of the reconstituted sample, k is the dilution factor, 5 is the dilution factor associated with the enzymatic reaction and protein precipitation steps, m is the sample weight, and 10 is the conversion factor expressing results in mg/100 g.

3.3. Validation of method

3.3.1. System suitability

System suitability testing confirmed that the LC-MS/MS system was stable and suitable for the quantitative analysis of difucosyllactose (DFL). Six replicate injections of a 10 ppm DFL standard produced consistent retention times of 8.39 ± 0.01 min, with a relative standard deviation (RSD) of 1.4% for peak area (mean peak area: 2155). These results demonstrate that the system met all suitability criteria and was suitable for subsequent method validation.

3.3.2. Specificity

The specificity of the method was confirmed by analyzing the DFL standard solution at a concentration of 4.00 µg/mL, blank samples, and samples with added standard from three different matrices, including infant formula, ready-to-feed milk, and probiotic yogurt at a standard addition level of 100 µg/g (**Figure 6**).

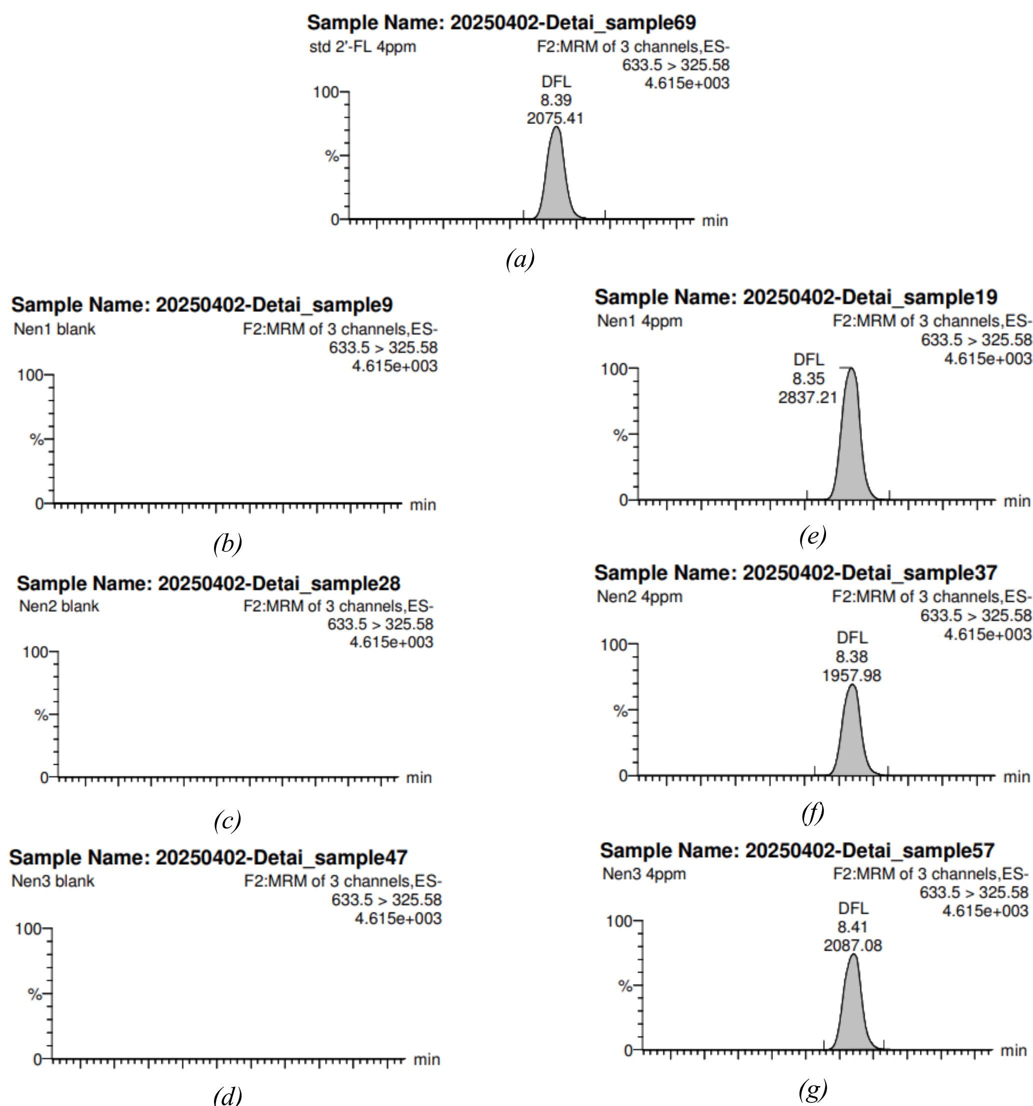

***Figure 6.** Chromatograms illustrating method specificity:*
(a) DFL standard solution; (b-d) blank samples of powdered infant formula, ready-to-feed milk and probiotic yogurt drink matrices; (e-g) spiked samples of the corresponding powdered infant formula, ready-to-feed milk and probiotic yogurt drink matrices

As illustrated in **Figure 6** show that no analyte signal was detected in the blank samples, while the retention time difference of DFL between the standard sample and the sample with added standard was less than 5%. Simultaneously, based on the MS conditions, the DFL achieved 5 IP points [22]. Therefore, the method meets the specificity requirements according to AOAC Appendix F.

3.3.3. Calibration curve

Based on the selected analytical conditions, a calibration curve for DFL was constructed by establishing a linear relationship between peak area and analyte concentration over the range of 1 - 20 µg/mL. The resulting calibration equation and the corresponding correlation coefficient are presented in **Table 1** and **Figure 7**.

The results indicate that the calibration curve for DFL exhibited a correlation coefficient $R^2 > 0.995$, with deviations at all calibration points below 15%. Therefore, a linear relationship between peak area and the corresponding DFL concentration was observed over the examined concentration range, which is consistent with the requirements specified in AOAC SMPR® 2021.006 and AOAC Appendix F, confirming the suitability of the method for DFL quantification.

Table 1. *Calibration curve parameters for DFL*

C (µg/mL)	Area	C' (µg/mL)	Bias (%)
1.00	451	0.97	-3.34
2.00	1078	2.03	1.48
4.00	2294	4.09	2.30
10.0	5829	10.1	0.89
15.0	8568	14.8	-1.77
20.0	11724	20.1	0.44

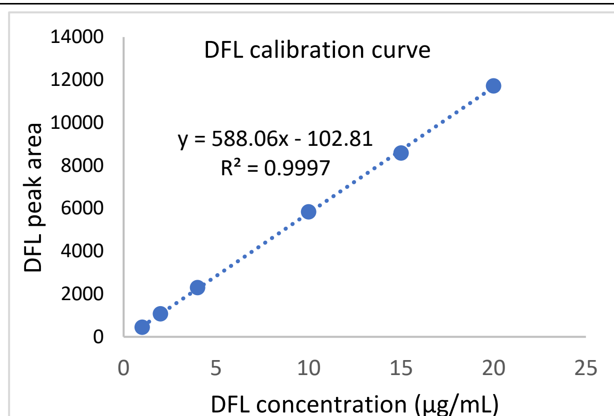

Figure 7. *Calibration curve of DFL*

3.3.4. Method detection limit (MDL) and method quantification limit (MQL)

The method detection limit (MDL) and method quantification limit (MQL) were determined by analyzing spiked blank samples at low concentration levels in ten replicate measurements across three different matrices, infant formula, ready-to-feed milk and probiotic yogurt drink, at three concentration levels. The mean value ($\bar{x}$) and standard deviation (SD) were calculated from the obtained data. MDL and MQL were defined as 3SD and 10SD, respectively. The estimated MDL and MQL values were further evaluated using the ratio $R\ value = \text{MDL}/\bar{x}$. The results are summarized in **Table 2.**

Table 2. *MDL and MQL results*

Matrix	MDL (µg/g)	MQL (µg/g)	R
Infant formula	4.80	16.0	5.20
Ready-to-feed milk	3.35	11.2	7.35
Probiotic yogurt drink	5.12	17.1	4.68

The R values obtained for infant formula, ready-to-feed milk, and probiotic yogurt drink matrices were within the range of 4 - 10. Therefore, the calculated limits of detection (MDL) and limits of quantification (MQL) were acceptable and met the performance requirements specified in SMPR® 2021.006.

3.3.5. Precision and accuracy

Method accuracy was evaluated based on recovery (Recovery%), while repeatability was assessed using the relative standard deviation under repeatability conditions (RSDr%). Intermediate precision was evaluated by two analysts on two different days and expressed as the relative standard deviation under intermediate precision conditions (RSD_R%). These parameters were determined by analyzing matrix blank samples spiked

with the analyte at three concentration levels (25, 100 and 500 µg/g), corresponding to analytical solution concentrations of 1, 4, and 20 µg/mL, respectively, across three different matrices, including infant formula, ready-to-feed milk, and probiotic yogurt. Each concentration level was analyzed in six replicates following the selected sample preparation procedure described in Section 3.1. The results of the repeatability and intermediate precision evaluation are summarized in **Table 3**.

Table 3. *Results of precision and recovery*

Matrix	Amount (µg/g)	Precision		Accuracy
		RSD_r (%)	RSD_R (%)	Recovery (%)
Infant formula	25.0	2.86	5.74	87.2 - 105
	100	2.47	3.87	92.2 - 106
	500	3.02	2.83	96.5 - 105
Ready-to-feed milk	25.0	3.59	4.65	90.9 - 104
	100	3.82	4.21	92.4 - 105
	500	2.87	4.67	91.9 - 105
Probiotic yogurt drink	25.0	3.92	5.25	93.2 - 109
	100	3.84	4.31	92.1 - 108
	500	3.93	3.45	94.1 - 105

Based on the results presented in **Table 3**, the method demonstrated precision and accuracy fully compliant with the performance requirements of AOAC SMPR® 2021.006. Specifically, the repeatability (RSD_r) of DFL in all three matrices (infant formula, ready-to-feed milk and probiotic yogurt drink) was below 5%, meeting the specified criterion for repeatability. The intermediate precision (RSD_R) values were all below 10%, satisfying the requirement for reproducibility.

In addition, the recoveries ranged from 87.2% to 109%, which fall within the acceptable range of 85 - 110% for the examined concentration levels as defined by the SMPR. These results confirm that the method exhibits good precision and accuracy, ensuring reliable quantification of DFL in milk-based and nutritional formula products.

3.3. Results on the analysis of real samples

The validated method was applied to the analysis of difucosyllactose (DFL) in 24 milk-based products supplemented with human milk oligosaccharides (HMOs), comprising 14 infant formulas, 7 ready-to-feed milks, and 3 probiotic yogurt drinks. The analytical results are shown in **Figure 8** and **Table 4**.

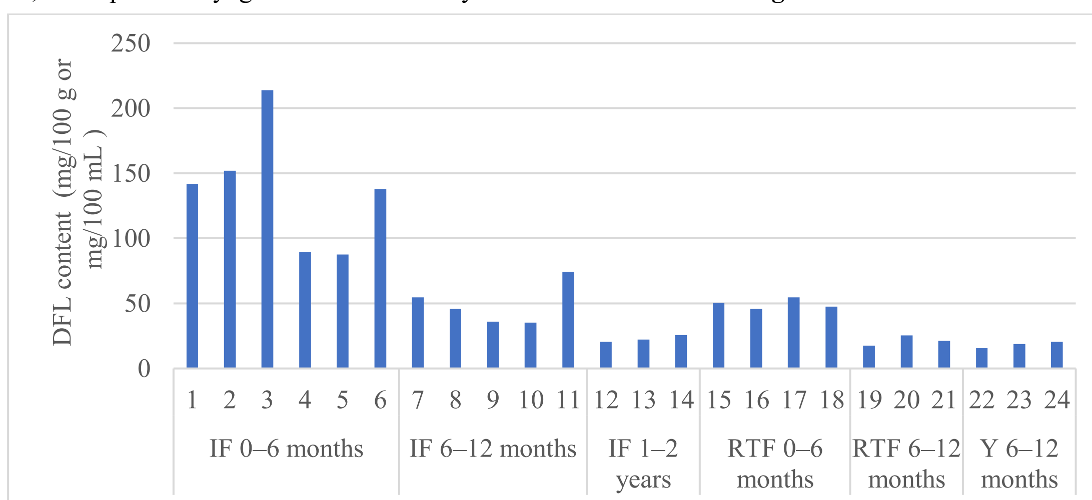

Figure 8. *DFL content levels in analyzed samples*

Table 4. *DFL and Label-declared HMOs contents levels in analyzed samples*

Matrix	Intended age group	Sample	DFL content (mg/100 g)	Label-declared HMOs content (mg/100 g)	DFL/ Total HMOs (%)
Infant formula	0 - 6 months	1	142	1350	10.5
		2	152	1500	10.1
		3	214	1800	11.9
		4	89.5	1500	5.97
		5	87.5	1500	5.83
		6	138	1400	9.86
	6-12 months	7	54.6	500	10.9
		8	45.9	410	11.2
		9	36.1	650	5.55
		10	35.2	650	5.42
		11	74.2	1400	5.30
	1 - 2 years	12	20.6	410	5.02
		13	22.3	435	5.13
		14	25.6	256	10.0
Ready-to-feed milk	0 - 6 months	15	50.6	510	9.92
		16	45.9	410	11.2
		17	54.6	525	10.4
		18	47.5	480	9.90
	6 - 12 months	19	17.6	340	5.18
		20	25.3	525	4.82
		21	21.3	450	4.73
Probiotic yogurt drink	6 - 12 months	22	15.6	258	6.05
		23	18.9	352	5.37
		24	20.6	410	5.02

The analytical results revealed pronounced differences in difucosyllactose (DFL) content across product matrices and targeted age groups. Powdered infant formulas exhibited the highest DFL levels, particularly in products formulated for infants aged 0 - 6 months (87.5 - 214 mg/100 g). A decreasing trend was observed in formulas intended for older age groups, with concentrations of 35.2 - 74.2 mg/100 g for infants aged 6 - 12 months and 20.6 - 25.6 mg/100 g for children aged 1 - 2 years.

In ready-to-drink nutritional milk products, DFL concentrations were substantially lower than those observed in powdered formulas, ranging from 45.9 to 54.6 mg/100 g for the 0-6 month age group and decreasing to 17.6 to 25.3 mg/100 g for the 6 - 12 month age group. Probiotic yogurt drinks, evaluated only for children aged 6 - 12 months, exhibited the lowest DFL levels (15.6 to 18.9 mg/100 g). Overall, the observed decrease in DFL content with increasing target age is consistent with previously reported changes in human milk oligosaccharide (HMOs) composition during lactation [24, 25].

Evaluation of the proportion of DFL relative to the total HMOs declared on product labels indicated that DFL typically accounted for approximately 5 - 10% of the declared HMOs content, which is consistent with the compositional profiles reported by HMOs ingredient suppliers [5, 26, 27]. These findings highlight the importance of DFL analysis for verifying product formulations, assessing label claims related to HMOs, and ensuring the nutritional appropriateness of milk-based products intended for infants and young children.

4. CONCLUSION

This study successfully developed an LC-MS/MS method for the determination of difucosyllactose (DFL) in powdered infant formula, ready-to-feed milk, and probiotic yogurt drink matrices with high sensitivity and short analysis time. The method demonstrated good specificity, low detection limits, and satisfactory accuracy and precision, meeting the requirements of AOAC (2016) and SMPR® 2021.006. The validated method was

applied to analyze 24 commercial milk products collected from the Hanoi market, Vietnam. The results indicated that DFL levels varied among different product matrices and targeted age groups; notably, five samples exhibited DFL concentrations below the limit of quantification (MQL). Overall, the developed LC-MS/MS method represents a reliable and effective analytical tool for DFL determination, with strong potential for quality control, verification of HMOs-related label claims, and consumer protection. To enhance the method's applicability, the team will validate its accuracy against common HMO compounds and compare the results with established standards to support food quality control.

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