Analyte	Intra-day precision at			Intra-day precision on		Inter-day precision at				Inter-day precision on		Averaged accuracy on		
	6*STD1 level	40*STD1 level	150*STD1 level	powdered milk A	powdered milk B	endogenous level	6*STD1 level	40*STD1 level	150*STD1 level	powdered milk A	powdered milk B	all levels	powdered milk A	powdered milk B
	[%] (n=6)	[%] (n=8)	[%] (n=8)	[%] (n=6)	[%] (n=6)	[%] (n=6)	[%] (n=6)	[%] (n=7)	[%] (n=7)	[%] (n=6)	[%] (n=6)	[%]	[%]	[%]
Thiamine	1.2	0.3	3.8	0.9	0.5	3.9	3.1	2.5	6.7	1.6	1.8	101.7	98.3	115.7
TMP	14.8	4.3	3.8	--	--	13.1	16.9	7.6	6.7	--	--	98.7	--	--
Riboflavin	2.8	2.8	1.9	1.5	1.2	2.7	3.0	4.0	2.5	1.9	1.3	102.9	112.1	96.0
FAD	16.0	6.0	1.4	--	--	6.8	18.5	8.5	5.1	--	--	95.2	--	--
FMN	14.9	4.4	1.9	--	--	12.7	12.7	4.1	3.2	--	--	95.1	--	--
Nicotinamide	3.1	1.5	1.4	1.2	0.7	1.8	3.5	2.3	2.5	1.3	1.1	98.5	93.7	99.8
Nicotinic acid	0.6	1.1	10.1	--	--	3.0	0.9	1.6	10.2	--	--	97.9	--	--
Nicotinuric acid	7.9	2.1	2.4	--	--	30.5	10.7	2.9	2.5	--	--	101.3	--	--
Nudifloramide	9.5	7.9	2.2	--	--	5.5	15.1	8.7	3.9	--	--	100.3	--	--
Pyridoxine	7.3	1.0	0.7	1.2	0.8	2.2	5.5	1.3	1.0	1.8	1.5	93.8	96.3	104.3
Pyridoxamine	3.2	0.5	0.9	--	--	3.8	3.3	1.1	0.9	--	--	99.2	--	--
Pyridoxal	7.9	1.5	2.5	--	--	2.9	9.9	5.7	3.5	--	--	107.5	--	--
PLP	5.7	3.4	7.7	--	--	12.2	13.7	9.6	9.2	--	--	92.2	--	--
PMP	8.3	8.2	10.3	--	--	15.3	10.9	8.1	11.7	--	--	92.9	--	--
4-Pyridoxic acid	28.3	24.4	9.7	--	--	15.4	32.6	25.5	8.1	--	--	102.8	--	--
Folic Acid	3.0	2.3	1.6	2.4	2.2	6.5	4.3	3.1	2.2	2.6	2.5	105.8	106.1	128.9
5-Me THF	5.4	2.2	2.9	--	--	4.9	6.2	2.1	2.7	--	--	99.0	--	--
p-ABGA	9.1	3.1	3.2	--	--	61.0	9.0	4.0	4.0	--	--	99.3	--	--
Average	8.3	4.3	3.8	1.4	1.1	11.3	10.0	5.7	4.8	1.9	1.7	99.1	101.3	108.9

Table 3: Performance parameters for the quantification of water soluble vitamers

Analyte	Averaged accuracy at					
	FT 0 [%]	FT 1 [%]	FT 2 [%]	FT 3 [%]	FT 4 [%]	FT 5 [%]
Thiamine	99.3	103.3	104.0	113.2	104.4	115.8
TMP	97.8	99.1	82.2	102.0	90.2	69.3
Riboflavin	104.0	104.2	106.2	117.1	114.0	125.4
FAD	98.9	91.9	85.8	105.2	61.9	125.0
FMN	99.6	122.5	124.6	163.1	138.6	174.0
Nicotinamide	99.1	98.1	99.1	103.8	99.3	116.4
Nicotinic acid	99.4	98.8	101.2	109.6	100.3	111.6
Nicotinuric acid	98.3	98.2	102.7	102.0	98.3	106.0
Nudifloramide	97.5	97.7	98.3	102.1	101.4	99.8
Pyridoxine	98.9	98.6	101.7	100.8	97.4	112.8
Pyridoxamine	99.5	89.3	104.5	104.5	115.7	122.6
Pyridoxal	103.4	106.4	106.9	105.1	103.3	123.0
PLP	102.2	97.4	99.9	82.4	53.4	64.8
PMP	95.5	104.2	124.9	163.9	167.9	231.2
4-Pyridoxic acid	106.9	109.0	105.7	115.9	87.0	152.0
Folic Acid	98.0	94.5	93.3	104.1	91.3	59.1
5-Me THF	100.4	72.2	35.5	4.3	4.6	8.8
p-ABGA	96.2	101.4	118.2	119.1	108.1	123.5

Table 4: Freeze/Thaw stability of water soluble vitamers

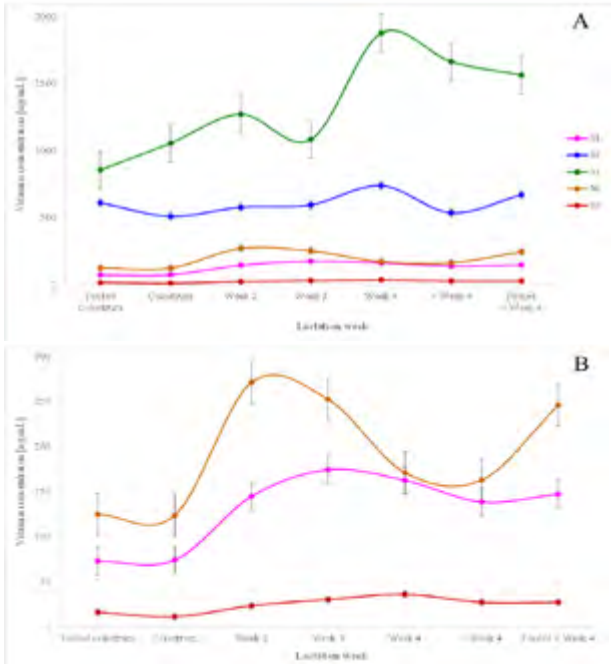

Figure 3: Breast milk kinetic profile of 5 B-vitamins (sum of related vitamers for each class) over the first weeks of lactation (from colostrum to mature milk) with B_2 and B_3 (A) in the 500-2000 ng/mL range and B_1, B_6 and B_9 in the 0-300 ng/mL range.

products. Rat serum volume was evaluated by applying the procedure to commercially available standard (5-Me THF diglutamic acid) at various concentrations (data not shown). Samples with big dynamic range (>factor 2) may be affected with rat serum treatment. However, at 1 to 5 ng/mL concentration conversion efficiency seemed to be similar (67% versus 64%). In terms of rat serum volumes, 10 µL seem to be sufficient to cleave almost all diglutamic acid units. Optimal conditions were defined as incubating BM samples after α-amylase treatment with 10 µL of rat serum during 2 hours at 37°C. BM samples were then treated with and without deconjugase. None of the B_9 derivative concentration significantly increased after deconjugase treatment. This suggests that 5-Me THF polyglutamate amounts in BM are negligible. However, we may suggest that the presence of antioxidants in the extraction media was desirable when extraction involves longer incubation times as published earlier [38].

Application to BM samples

BM samples underwent the sample preparation process and were quantified with the built-in calibration curves (prepared with pooled BM). Each vitamer was quantified individually with its dedicated calibration curve and then concentrations were summed up to obtain the final concentration of each B-vitamin. Figure 3 highlights the evolution of each of the above listed B-vitamin along the 4 first weeks of lactation.

In the early 2000, thiamine concentration was reported as vitamin B_1 level in breast milk [15,39]. However, in the recent years, the sum of thiamine and TMP seems to best represent total B_1 vitamin levels in breast milk [21,30,40] which was also applied in our work as both compounds were quantified in our work. Indeed, the main secreted form of B_1 in breast milk is thiamine monophosphate, contributing for about 60% of total B_1 [21,30,40] confirmed in our findings whereas thiamine is the compound used for fortification/supplementation. B_1 concentration increases in the transition milk (week 2 to week 4) to reach a steady level around 150 ng/mL in mature milk (>4 weeks).

B_2 concentration was obtained by adding riboflavin, FAD and FMN concentrations. Vitamin B_2 main secreted form in breast milk is FAD, contributing for at least 60% total B_2 [18]. As reported in previous findings [18,39], our results on vitamin B_2 emphasized on the importance of quantifying not only riboflavin but also flavin derivatives to best quantify total B_2 in breast milk.

Nicotinamide concentration was previously reported as vitamin B_3 level in BM [28,39]. Our findings confirm that nicotinamide is the primary B_3 vitamer present in BM, however we additionally show that nudifloramide (N-methyl-2-pyridone-5-carboxamide) is also present at a similar level compared to nicotinamide. These findings have never been reported before. We also included nicotinic acid and 1 metabolite nicotinuric acid known to be present in urine [34]. B_3 concentration increases in the transition milk (week 2 to week 4) to reach a steady level around 1600 ng/mL in mature milk (>4 weeks).

B_6 concentration was obtained by adding pyridoxine, pyridoxamine, pyridoxal, PLP, PMP and 4-pyridoxic acid concentrations. Main secreted form of vitamin B_6 in BM was reported to be PL [28,41-44], which has been confirmed in our findings. Among all B_6 vitamers, PL contributed to about 75%, PLP to about 15% and only traces for all other forms which is in agreement with previous findings from Hamaker et al. [42,44]. In addition, our findings revealed the presence of 4-pyridoxic acid, which confirms previous findings [45]. Its content reached about 20 ng/mL representing about 5% of total vitamin B_6.

B_9 concentration was obtained by adding folic acid, 5-Me THF and *p*-ABGA concentrations. Our results show that the main secreted form was found to be folic acid, contributing to about 75% of total vitamin B_9. The presence of polyglutamate forms was investigated but our findings go against previous findings [46]. O'Connor et al. [46] quantified folates by microbiological assay. They applied conjugase treatment to liberate potential polyglutamate forms of folates and quantified total folates after 6 h incubation. The increase in folate amounts in breast milk after deconjugase O'Connor observed is probably due to other existing forms such as 5-formyl THF. In our study, we only focused on the quantification of 5-Me THF in addition to folic acid. Our findings support the recently reported B_9 derivative *p*-ABGA [47] in BM. B_9 concentration triples from week 1 to week 4 to reach a steady level at about 25 ng/mL whereas B_2 concentration seems to be stable around 600 ng/mL along the first lactation weeks. B_6 concentration doubles from week 1 to 2 but decreases afterward.

The calibration ranges described in this paper are comparable to the ones reported previously by Hampel et al. [28] and Ren et al. [29]. Our methodology, despite the use of 200 µL of BM as opposed to only 50 µL used by Hampel et al. [28] and Ren et al. [29], allows for the quantification of TMP, being the main secreted form for B_1 evaluation. Measurement of all secreted vitamers will provide a more comprehensive picture of the B-vitamin levels in breast milk to get insight into nutritional needs of the breastfed infants.

Conclusion

This analytical approach based on protein precipitation combined with liquid chromatography-tandem mass spectrometry measurements enabling the accurate quantification of 18 water soluble vitamers in breast milk was successfully developed and validated. Matrix-matched

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