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A Systematic Review of Collection and Analysis of Human Milk for Macronutrient Composition

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ABSTRACT

Background: As human milk (HM) composition varies by time and across even a single feed, methods of sample collection can significantly affect the results of compositional analyses and complicate comparisons between studies.

Objective: The aim was to compare the results obtained for HM macronutrient composition between studies utilizing different sampling methodologies. The results will be used as a basis to identify the most reliable HM sampling approach.

Methods: EMBASE, MEDLINE/PubMed, Cochrane Library, Scopus, Web of Science, and ProQuest databases were searched for relevant articles. Observational and interventional studies were included, and at least 2 authors screened studies and undertook data extraction. Quality assessment was conducted using the Newcastle-Ottawa scale and previously published pragmatic score.

Results: A total of 5301 publications were identified from our search, of which 101 studies were included (n = 5049 breastfeeding women). Methods used for HM collection were divided into 3 categories: collection of milk from all feeds over 24 h (32 studies, n = 1309 participants), collection at one time point (62 studies, n = 3432 participants), and "other methods" (7 studies, n = 308 participants). Fat and protein concentrations varied between collection methods within lactation stage, but there were no obvious differences in lactose concentrations. There was substantial variability between studies in other factors potentially impacting HM composition, including stage of lactation, gestational age, and analytical method, which complicated direct comparison of methods.

Conclusions: This review describes the first systematic evaluation of sampling methodologies used in studies reporting HM composition and highlights the wide range of collection methods applied in the field. This information provides an important basis for developing recommendations for best practices for HM collection for compositional analysis, which will ultimately allow combination of information from different studies and thus strengthen the body of evidence relating to contemporary HM composition. This trial was registered at PROSPERO as CRD42017072563, https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42017072563 *J Nutr* 2020;150:1652–1670.

Keywords: systematic review, human milk composition, macronutrients, breast milk collection, infant health

Introduction

Human milk (HM) is uniquely designed for the human infant, containing the nutrients and bioactive components that are required to support optimal growth and development, and thus has an important role in infant survival and health (1). Consequently, the WHO recommends that infants should be exclusively breastfed until 6 mo postpartum, with continued breastfeeding to 2 y of age or beyond (2). It is critical to understand contemporary HM composition given the established importance of nutritional exposures in early infancy for an individual's lifelong health outcomes, reinforced by significant relations between the concentration of specific components in HM and both short-term infant growth/body composition and future risk of obesity, metabolic disorders, and other noncommunicable diseases (3, 4).

Despite recognition of the importance of breastfeeding and HM, research in this area is complicated by the fact that HM composition is highly variable and can change according to the time of the day and stage of lactation, as well as between women and populations (5–8). The method and time of sample collection also have the potential to significantly influence HM composition, including the concentrations of macronutrients (fat, protein, and lactose, which ultimately impact energy content), as well as concentrations of other bioactive factors in

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HM (5, 6, 9). For example, the fat concentration in HM can increase up to 3 times from its initial concentration across a single feed from 1 breast, and also increases progressively across the course of a day (10).

The sub-sampling and pooling of milk samples from a full breast expression at each feed across a 24 h period are considered the "gold standard" for HM collection (11, 12). However, this approach places a significant burden on the mother, is not possible in certain groups, and is impractical for population studies in which the goal is to obtain compositional information in a representative sample of women. Currently, several collection methods are utilized by different research groups, and there is no universal, standardized sampling approach. Thus, it is important to establish a reliable and practical method of HM collection that best represents the average composition of the milk that is being consumed by the infant, while ensuring that the method is practical, feasible, and has minimal interference with normal breastfeeding. To date, it is unclear which of the existing collection methods provide a measure of HM composition that is most closely aligned to the "gold standard" approach of 24 h pooled collection. The aim of this systematic review was to compare the results obtained for HM macronutrient composition between studies utilizing different sampling methodologies. The results will be used as a basis to identify the most reliable HM sampling approach.

Methods

Protocol

The systematic review protocol was developed based on Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (PRISMA-P) guidelines (13, 14) and the Cochrane Handbook for Systematic Reviews of Interventions (15). The full version of the protocol and further details, including PRISMA-P checklist file, has been previously published (16). The search strategy for MEDLINE/PubMed is presented in **Supplemental Table 1**. This trial was registered at PROSPERO as CRD42017072563.

Eligibility criteria

To be eligible for inclusion, studies must have included women who were currently breastfeeding (exclusively or partially) or routinely expressing HM (manually or using a breast pump). The studies could be conducted at any lactation stage and had to have reported the time of day of milk collection, the method of collection (i.e., whether samples were collected pre-feed, post-feed, or a full expression) and ≥1 measure of HM macronutrient composition (total protein, total fat and/or lactose). Studies not reported in the English language or only reported as abstracts were not included.

Information sources and search strategy

Literature searches were undertaken using the following electronic bibliographic databases: EMBASE, MEDLINE/PubMed, Cochrane Library, Scopus, Web of Science, ProQuest Dissertations, and Thesis Global. The literature search was limited to studies in humans, but no date range restrictions were applied, and the last search was conducted in January 2018.

Selection process

The selection of articles for inclusion in the review was undertaken in 2 stages. The first stage involved screening the title and abstracts of the search results against the eligibility criteria. In the second stage, the full articles of papers selected in the title/abstract screening stage were screened to confirm that they met the eligibility criteria. At both stages, each article was screened independently by 2 authors. In the case of disagreement as to the eligibility status between the first 2 authors, the study was reviewed by a third author and any disagreements were resolved by mutual discussion.

Data extraction

Two authors independently extracted data from each included study based on a standardized extraction form modified from the Cochrane Pregnancy and Childbirth Group (17). Data extracted included methodological details [e.g., stage of lactation and time of day when milk was collected, method of collection, and analytical method for the reported macronutrient(s)], gestational age, mode of feeding (exclusively breastfeeding or mixed feeding), mode of collection (hand expression, manual/electric breast pump, or both), time since last feeding, volume collected per sample, number of samples collected per participant, and total number of samples analyzed in the study. In addition, information was extracted as to whether measures of maternal milk production or infant intake were undertaken in the study, including the use of test weighing (weighing infant before and after each feeding for 24 h), weighing of mothers before and after each feed, estimates from 24 h volume output, or use of deuterium oxide dilution technique. When missing data were identified, efforts were made to contact the corresponding authors and co-authors of the relevant study (maximum of 3 attempts).

Risk of bias in individual studies

Quality assessment of each included article was conducted independently by 2 authors based on the Newcastle-Ottawa scale and on a pragmatic score adapted from Andreas et al. (18). The risk of bias assessed the representativeness of the cohort ("truly represents" and "somewhat represents" the average lactating women in the community) and whether the study controlled for confounding factors (including, but not limited to, maternal and infant age, infant sex, and stage of lactation when the sample was collected). Based on the previously published pragmatic score, we also included additional categories: sample size (small = studies with <50 participants, medium = studies with between 50 and 100 participants, and large = studies with >100 participants); whether the study *1*) stated feeding mode (exclusively or partially breastfeeding); *2*) standardized stage of lactation when milk sample/s were collected; *3*) stated mode of HM collection (manual expression and/or breast pump); *4*) stated gestational age of infants; and *5*) reported the analytical method used for macronutrient analyses. In the scoring matrix, studies were awarded a maximum of 1 point per category for binary questions (yes = 1, no = 0). For the sample size question, small, medium, and large studies were assigned 0.5, 1, and 1.5, points respectively. As a result, studies received a score out of a possible 8.5. The quality-assessment scores for each collection method are presented as means ± SDs. Other categories, including time since last feed and use of HM as the standard for protein analysis, were also included in the risk of bias assessment when these questions were applicable, but these data are presented separately since they were not relevant for all included studies.

Data synthesis

As the included data were highly diverse, it was not possible to conduct a meta-analysis and therefore findings are presented in the form of structured tables for each macronutrient (total protein, total fat, and lactose). Macronutrient concentrations are reported in grams per liter,

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Supplemental Tables 1–5 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.
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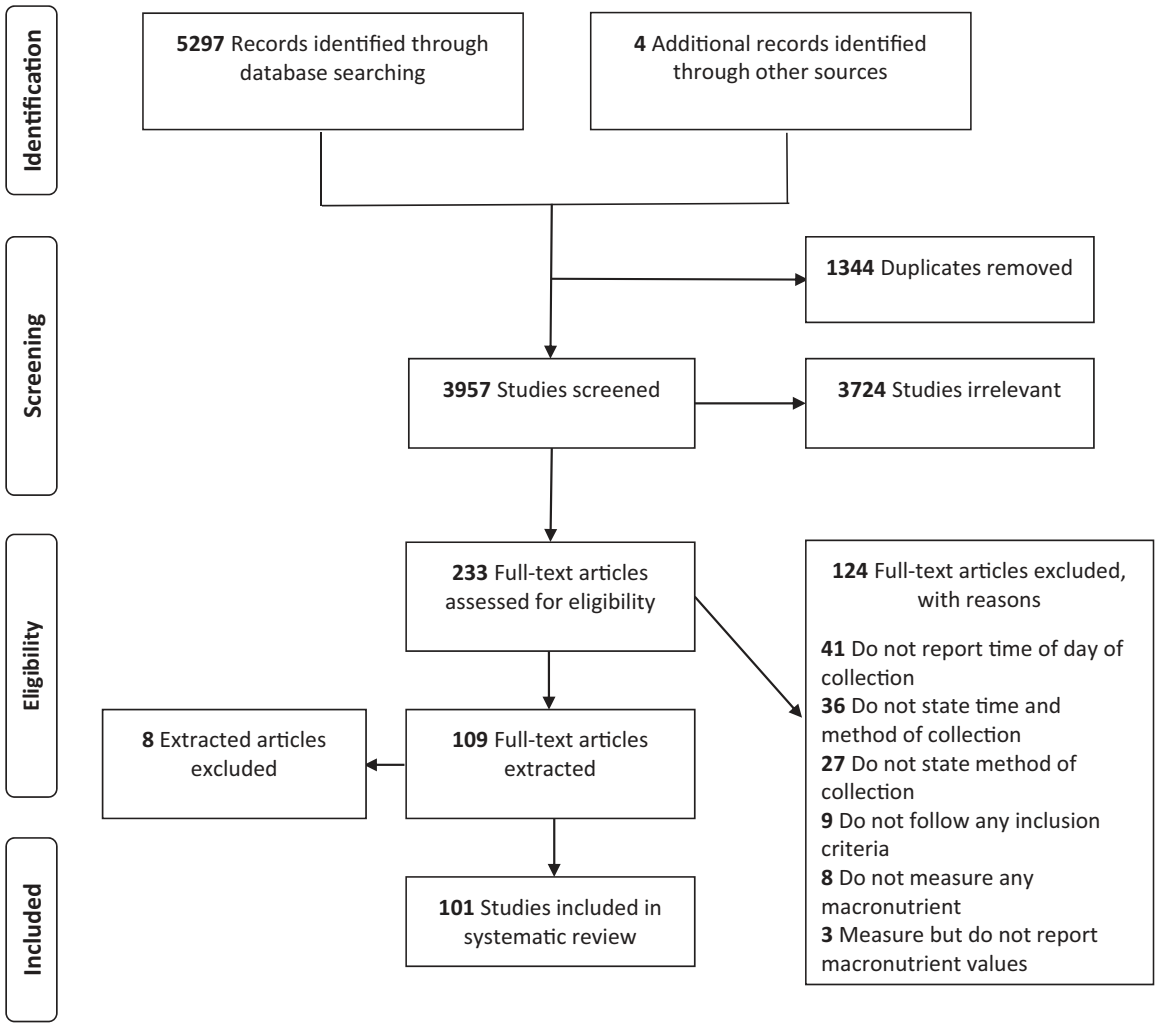

FIGURE 1 PRISMA flow diagram summarizing the process of article screening and reasons for exclusion. PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analysis.

and any results reported in other units were first converted to grams per liter to allow direct comparison between studies. Concentrations that were reported as either percentage of creamatocrit (for fat) or total nitrogen (for protein) were converted to fat and total protein concentrations using standard conversion conventions (19) and formulas (20–22). Fat concentrations calculated from the cream percentage were based on the equation: 5.917 × cream percentage + 3.968 (19). For studies that only reported total nitrogen concentration, we considered the nonprotein nitrogen to represent 20% of this value (23, 24). Total protein was calculated based on the equation: protein nitrogen = total nitrogen − non protein nitrogen; protein nitrogen × 6.28 = total protein (20, 21). For the purpose of this systematic review, triglyceride concentrations were considered to be equivalent to fat concentration, and carbohydrate concentration was considered to be equivalent to lactose, since triglyceride makes up ~98% of the total lipids and lactose makes up ~98% of the total carbohydrates in HM (25, 26).

To provide a synthesis of macronutrient composition of HM at different lactation stages, mean macronutrient concentrations in colostrum, transitional, and mature milk were calculated for studies meeting specific inclusion criteria. These studies were conducted in term infants, clearly defined the stage of lactation when samples were collected, and reported analysis of HM samples collected at that stage (not pooled samples from different lactation stages). In the case of studies where samples were collected at one time point in the day, sample collection was conducted in the morning, as this was the most common approach.

Results

Summary of studies

Our search strategy identified a total of 5301 publications, of which 101 were included in this systematic review (n = 5049 breastfeeding women) A total of 8 studies were excluded after data extraction for reasons shown in **Supplemental Table 2**. Figure 1 provides the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) diagram for the search.

A summary of the included studies is presented in Table 1. The included studies were conducted in 38 different countries and regions, with sample sizes ranging from 1 to 156. Most of the studies were conducted in the United States (n = 31, n = 1055 women), followed by Australia (n = 11, n = 267 women). The majority of the studies (n = 89) were observational cohort studies, with 56 being longitudinal and 33 being cross-sectional in design. The remaining studies were interventional (n = 9) or case studies (n = 3). The studies included in this review had a wide range of publication dates, between 1959 and 2018, however, all but 3 studies (27–29) were published after 1980.

Samples were collected across a broad range of postpartum ages, from the day of birth to 26 mo postpartum, with most

TABLE 1 Summary of studies of human milk composition included in the systematic review[1]

Reference	Site	Sample size	Stage of lactation	Outcomes measured		
				Milk fat or TGs	Milk protein or nitrogen	Milk lactose or carbohydrates
Milk samples collected across 24 h						
Full expression across 24 h						
Anderson et al., 1983 (30)	USA	23	3, 7, and 14 d	✓	✓	✓
Beijers et al., 1992 (31)	Netherlands	45	1 wk and weekly/biweekly (consecutive samples)		✓	
Bishara et al., 2008 (32)	Canada	24	21–30 d		✓	
Boutte et al., 1985 (33)	USA	9	— (average 3.2 mo)	✓		
Brown et al., 1982 (34)	Bangladesh	7	— (infants aged 6–29 mo)	✓	✓	✓
Brown et al., 1986 (35)	Bangladesh	58	0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 mo	✓	✓	✓
Butte et al., 1984 (36)	USA	45	1, 2, 3, and 4 mo	✓	✓	✓
Cregan et al., 2002 (37)	Australia	38	5 d		✓	✓
Heinig et al., 1993 (38)	USA	73	3, 6, 9, and 12 mo	✓	✓	✓
Lovelady et al., 1990 (39)	USA	16	9–24 wk	✓	✓	✓
McCrory et al., 1999 (40)	USA	67	8–16 wk	✓	✓	
Motil et al., 1997 (41)	USA	22	6, 12, 18, and 24 wk	✓	✓	✓
Perez-Escamilla et al., 1995 (42)	Honduras	141	4, 5, and 6 mo	✓	✓	✓
Stafford et al., 1994 (43)	Mexico	10	—	✓		
Stellwagen et al., 2013 (44)	USA	19	—	✓	✓	✓
Stuff and Nichols, 1989 (45)	USA	45	4–9 mo (consecutive samples)	✓	✓	✓
Pre- and post-feed across 24 h						
Cannon et al., 2015 (9)	Australia	19	3–21 wk	✓	✓	✓
Daly et al., 1993 (46)	Australia	5	4–9 mo	✓		
Jackson et al., 1988 (47)	Thailand	25	3 wk–9 mo	✓		
Kent et al., 2006 (10)	Australia	71	1–6 mo	✓		
Khan et al., 2013 (6)	Australia	15	1–6 mo	✓	✓	✓
Mitoulas et al, 2002 (48)	Australia	17	1, 2, 4, 6, 9, and 12 mo	✓	✓	✓
Perrella and Geddes, 2016 (49)	Australia	1	4 mo	✓	✓	
Prentice et al., 1981 (50)	Gambia	60	1–18 mo	✓		
Saint et al., 1986 (51)	Australia	9	2–12 mo	✓	✓	✓
Valentine et al., 1994 (52)	USA	15	1–4 wk	✓	✓	
Post-feed across 24 h						
Agostoni et al., 2001 (53)	Italy	95	1 d; 1, 3, 6, 9, and 12 mo	✓		
Agostoni et al., 2003 (54)	Italy	92	1 d; 1, 3, and 6 mo	✓		
Bauer and Gerss, 2011 (55)	Germany	112	1, 2, 3, 4, 5, 6, 7, and 8 wk	✓	✓	✓
Marangoni et al., 2000 (56)	Italy	10	1, 3, 6, 9, and 12 mo	✓		
Marangoni et al., 2002 (57)	Italy	54	1 d and 3 mo	✓		
Morton et al., 2012 (58)	USA	67	Weekly from 1–8 wk	✓	✓	✓
Milk samples collected at one time point						
Full expression						
Aksit et al., 2002 (59)	Turkey	80	2 mo	✓		
Barbosa et al., 1997 (60)	Mexico	40	3 and 6 mo	✓	✓	✓

(*Continued*)

TABLE 1 (*Continued*)

Reference	Site	Sample size	Stage of lactation	Outcomes measured		
				Milk fat or TGs	Milk protein or nitrogen	Milk lactose or carbohydrates
Butte et al., 1984 (61)	USA	21	2, 4, 6, 8, 10, and 12 wk	✓	✓	
Butte et al., 1992 (62)	Mexico	30	4 or 6 mo	✓	✓	✓
Chen et al., 1998 (63)	USA	40	5 d			✓
De Pee et al., 1997 (64)	Indonesia	155	3–6, 7–9, 10–12, and 13–18 mo	✓		
Dewey and Lönnerdal, 1983 (65)	USA	20	1, 2, 3, 4, 5, and 6 mo	✓	✓	✓
Foda et al., 2004 (66)	Japan	39	0–12 and 13–46 wk	✓	✓	✓
Goran et al., 2017 (67)	USA	25	1 and 6 mo			✓
Heon et al., 2016 (68)	Canada	40	1, 3, and 6 wk	✓		
Jackson et al., 1994 (69)	USA	77	2, 3, 7, 14, 42, and 84 d	✓		✓
Lönnerdal et al., 1976 (27)	Ethiopia	104	0–0.5, 0.5–1.5, 1.5–3.5, 3.5–6.5, 6.5 mo		✓	✓
	Sweden	–	4 and 5 d		✓	✓
Marquis et al., 2003 (70)	Peru	137	2 and 30 d	✓	✓	✓
Masters et al., 2002 (71)	USA	10	1–12 mo	✓		
Moran-Lev et al., 2015 (72)	Israel	32	1, 2, 3, 4, 5, 6, and 7 wk	✓	✓	✓
Mosley et al., 2007 (73)	USA	12	6–10mo	✓	✓	✓
Neubauer et al., 1993 (74)	USA	46	2, 3, 7, 14, 42, and 84d		✓	✓
Park et al., 1999 (75)	USA	16	1–26 mo	✓		
Perrin, 2015 (76)	USA	19	11, 12, 13, 14, 15, 16, and 17 mo	✓	✓	✓
Ritzenthaler et al., 2005 (77)	USA	44	1–10 mo	✓	✓	✓
Sauer et al., 2017 (78)	USA	24	1, 7, 14, and 21 d	✓	✓	✓
Szlagatys-Sidorkiewicz et al., 2013 (79)	Poland	156	17–30 d	✓		
Thurl et al., 1993 (80)	Germany	5	8 and 57 d			✓
Wack et al., 1997 (81)	USA	133	0–60, 61–120, 121–180, 181–240, 241–300, 301–360, >360 d			✓
Williams et al., 2017 (82)	USA	16	— (average 5 mo)	✓	✓	✓
Young et al., 2017 (83)	USA	56	2 wk and 4 mo	✓	✓	✓
Pre- and post-feed						
Da Cunha et al., 2005 (84)	Brazil	77	15 d	✓		
Fornes and Dorea, 1995 (85)	Brazil	39	15, 30, 45, 60, 75, and 90 d	✓		
Gridneva et al., 2017 (86)	Australia	27	2 and 5 mo	✓	✓	✓
Grote et al., 2016 (8)	Europe	30	1, 2, 3, and 6 mo	✓	✓	✓
Hahn et al., 2018 (87)	Korea	80	4 wk	✓	✓	✓
Hassiotou et al., 2013 (88)	Australia	6	6–39 wk (consecutive samples)	✓	✓	
Karatas et al., 2011 (89)	Turkey	46	1–3 and 4–6 mo	✓		
Kugananthan et al., 2017 (7)	Australia	59	2, 5, 9, and 12 mo		✓	✓
McDaniel et al., 1989 (90)	Not clear (USA or Canada)	24	2–20 mo	✓	✓	✓
Michaelsen et al., 1994 (91)	Denmark	91	4, 14, 28, 42, 56, 70, 84 d; 4, 5, 6, 7, and 8 mo	✓	✓	✓
Prentice et al., 1981 (92)	Gambia	120	1–18 mo (consecutive samples)	✓		
Schueler, 2011 (93)	USA	13	29–38 d	✓		
Pre-feed						
Al-Awadi and Srikumar, 2001 (94)	Kuwait[2]	34	0–6, 6–12, and 12–18 mo		✓	
Antonakou et al., 2013 (95)	Greece	64	20–30 d; 3 and 6 mo	✓		

(*Continued*)

TABLE 1 (*Continued*)

Reference	Site	Sample size	Stage of lactation	Outcomes measured		
				Milk fat or TGs	Milk protein or nitrogen	Milk lactose or carbohydrates
Bachour et al., 2012 (96)	Lebanon	66	10–365 d	✓	✓	
Chavalittamrong et al., 1981 (97)	Thailand	135	0–7, 8–14, 15–21, 22–28, 29–90, 91–180, 180–270, >270 d		✓	
Donangelo et al., 1989 (98)	Brazil	83	1–5, 6–30, and 31–280d		✓	
Dudzik et al., 2008 (99)	Poland	51	3, 21 and 100 d		✓	✓
Fujita et al., 2012 (100)	Kenya	83	0–20 mo		✓	
Khin-Maung et al., 1980 (101)	Burma	23	1–4, 4–7, and 7–12 mo	✓	✓	✓
Picciano and Guthrie, 1976 (28)	USA	50	6–12 wk	✓	✓	
Trugo et al.,1988 (102)	Brazil	59	1–5 and 6–36 d	✓	✓	
Tyson et al., 1976 (29)	Chile	9[3]	13–71 d (consecutive samples)	✓	✓	
Mid-feed						
Allen et al., 1991 (103)	USA	13	21, 45, 90, and 180 d	✓	✓	✓
Dutta et al., 2014 (104)	India	33	7, 28, 90, and 180 d	✓	✓	✓
Kon et al., 2014 (105)	Russia	103	1, 2, and 3 mo	✓	✓	
Lubetzky et al., 2015 (106)	Israel	72	3, 7, and 14 d	✓	✓	✓
Mandel et al., 2005 (107)	Israel	61	2–6 and >12 mo	✓		
Pines et al., 2016 (108)	Israel	57	1–7 mo	✓	✓	✓
Quinn, 2013 (109)	The Philippines	103	<18 mo	✓	✓	✓
Quinn et al., 2016 (110)	Nepal	82	—	✓	✓	✓
Post-feed						
Bener et al., 2001 (111)	United Arab Emirates	26	—	✓	✓	✓
Cant et al., 1991 (112)	Canada	36	2–6 and 10–14 mo	✓		
Moltó-Puigmartí et al., 2011 (113)	Spain	43	2–4, 8–12, and 28–32 d	✓		
Rakicioglu et al., 2006 (114)	Turkey	21	2–5 mo	✓	✓	✓
Vítolo et al., 1993 (115)	Brazil	136	1–2 d	✓	✓	
Milk samples collected by other methods						
Bassir, 1959 (116)	Nigeria	16	1, 2, and 3 mo; mixed mid-feed and post-feed	✓	✓	
Britton, 1986 (117)	USA	1	15 d; mixed pre-feed and mid-feed		✓	
De Luca et al., 2016 (118)	France	100	1 mo; expression lasted for as long as infant suckled	✓	✓	✓
Eilers et al., 2011 (119)	Germany	77	3 and 28 d; pumping for 10 min	✓	✓	
Neville et al., 1984 (120)	USA	18[4]	1–7 mo; mixed mid-feed and post-feed	✓	✓	✓
Shehadeh et al., 2006 (121)	Israel	84	3 mo or >12 mo; from 3 min until emptying the breast	✓	✓	✓
Woolridge et al., 1990 (122)	UK	12	4, 5, and 6 wk; pre-feed and post-feed for 12 h	✓		

[1]Every effort was made to follow a standardized approach when capturing information from studies into the summary tables. Any variation to the approach was due to the way in which the variable was reported in the respective paper. Methods of collection: studies were organized according to the method of collection described in the paper and the following definitions of HM methods of collection. Full expression: defined as milk collected when a single breast or both breasts were completely emptied. Pre-feed: defined as milk collected prior to infant feeding or milk collected for 3 min after milk flow began. Mid-feed: defined as milk collected at middle of the feeding or expression (after pre-feed and before post-feed). Post-feed: defined as milk collected at the end of the feeding or expression. Site: country/region where study participants were recruited. Sample size: number of participants who completed the study and provided milk samples considered for milk analyses according to our eligibility criteria. When this information was unavailable or unclear, we reported the number of individuals who participated in the study. Stage of lactation: period of lactation during which the milk samples were collected. If multiple samples were collected, e.g., on a weekly or daily basis, each time point or time window in lactation when samples were collected was reported (e.g., "3, 7 and 14 d postpartum" indicates that milk samples were collected at each of these time points, while "21–30 d postpartum" indicates that milk sample was collected between these time points and the study did not provide exact time point of collection). HM, human milk; TG(s), triglyceride(s); —, information not stated or unclear. The checkmark symbols indicate which macronutrient was assessed by each study.
[2]Nationalities of non-Kuwaitis group included American, Egyptian, Indian, Czech, and Taiwanese.
[3]Results only reported for study 3.
[4]Results only reported for protocol 2 and 3.

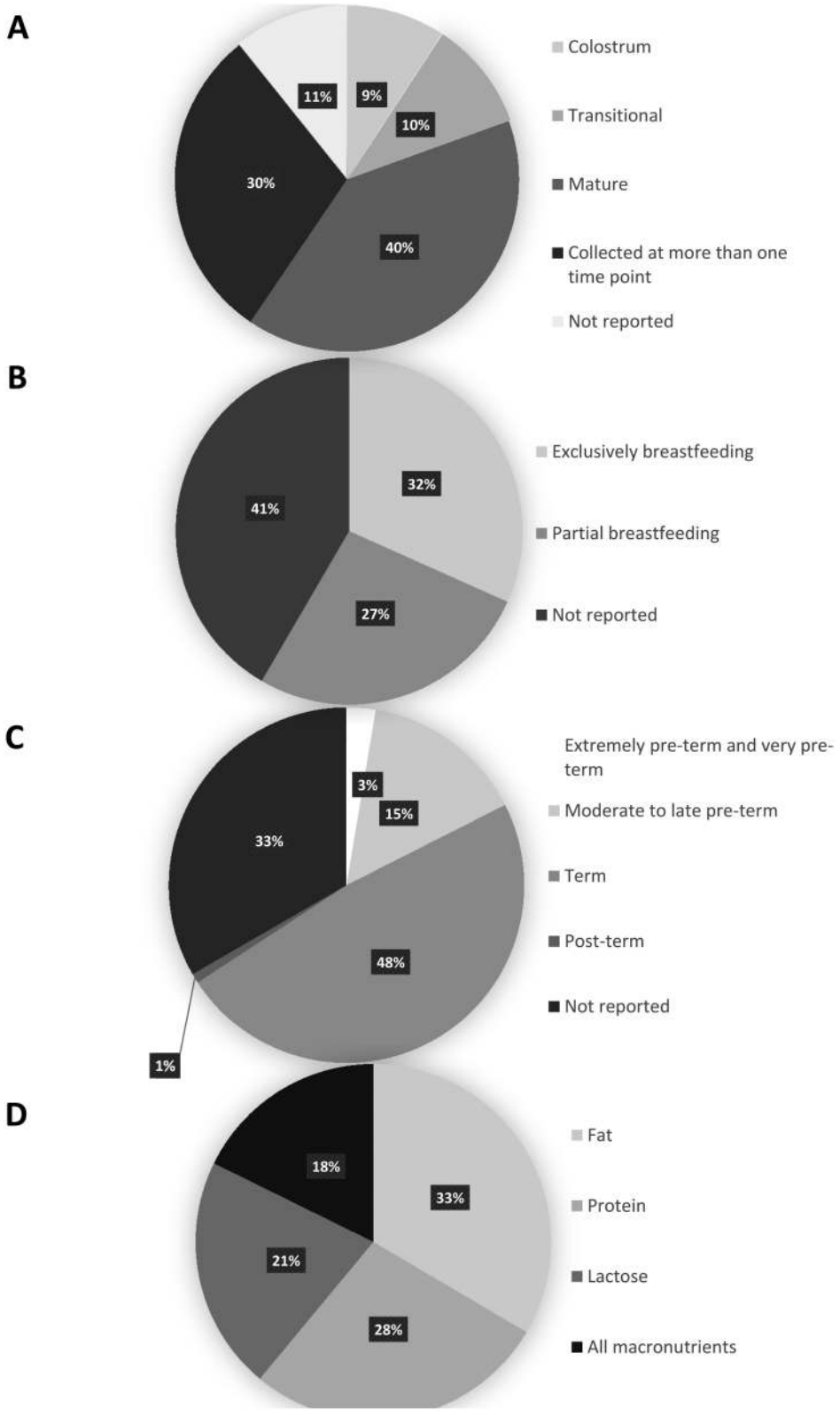

FIGURE 2 Distribution of included studies of human milk composition according to the type of milk collected (A), mode of feeding (B), gestational age of the infant (C), and milk macronutrient concentrations measured (D).

(78 studies) collecting mature milk and 58 studies collecting samples at more than one stage of lactation (**Figure 2**A). Of those studies that reported mode of feeding ($n = 59$), the number in which women were exclusively breastfeeding compared with partially breastfeeding was similar (Figure 2B). HM samples were collected from mothers of infants born at different gestational ages, ranging from extremely preterm (<28 weeks' gestation) to post-term (>42 weeks' gestation); however, the majority of studies were conducted in mothers of term infants (Figure 2C).

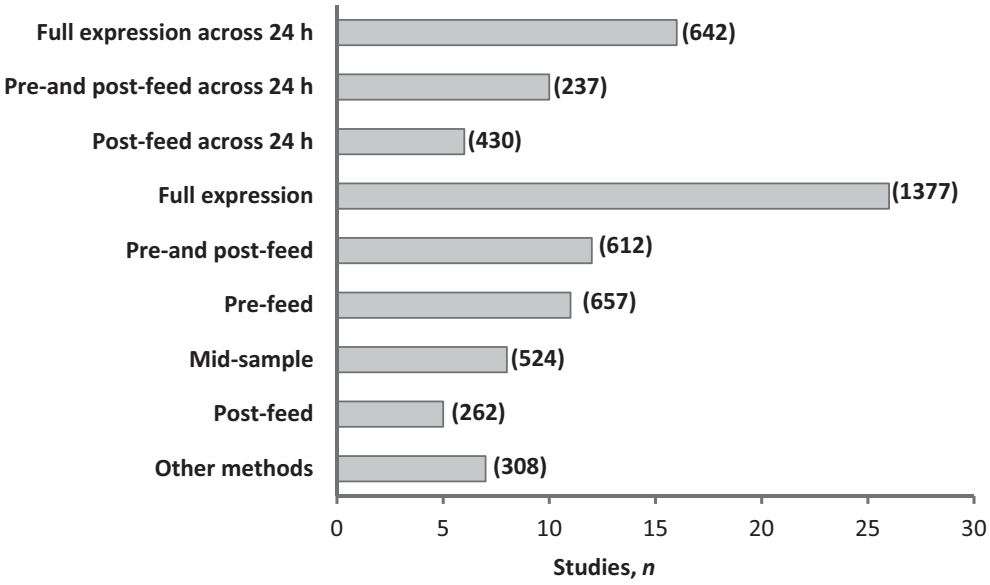

FIGURE 3 The number of studies applying each human milk collection method. Number of studies is depicted by the bar chart, with the corresponding number of participants shown in parentheses.

All studies included a measure of at least one macronutrient, and 45 studies included measures of all 3 macronutrients. Total fat was the most commonly measured macronutrient (85 studies), followed by total protein (70 studies) and lactose (54 studies) (Figure 2D).

Summary of methods used

The wide range of HM collection methods were divided into 3 broad categories, as follows:

1. Studies in which milk samples were collected at each feeding over a 24 h period (n = 32 studies, 1309 participants). This included studies in which either full expression, pre- and post-feed samples, or post-feed samples only collected at each feed over at least one 24 h period.
2. Studies in which milk samples were collected at one time point during the day (n = 62 studies, 3432 participants). This category was subdivided into studies in which a full expression, pre and post-feed samples, pre-fee samples only, mid-feed samples only, or post-feed samples only had been collected.
3. Studies in which milk samples were collected by other methods (7 studies, 308 participants), which included all studies whose method of collection did not fit into any of the other categories.

Additional details of the number of studies and participants in each of the sub-categories above are provided in **Figure 3**.

The most frequently used method was collection of a full expression at one time point, followed by pooling of full expressions of all feeds across 24 h and collection of pre- and post-feed samples at one time point. Of the studies collecting HM samples at one time point (n = 62), the majority (n = 47) collected samples only in the morning, followed by afternoon and evening samples (n = 7). Samples were collected at more than one time (n = 6) or the time of collection was unclear (n = 2) in 8 studies. Measures of maternal milk production and/or infant milk intake were measured by 46 studies. Of these, most collecting HM samples across a 24 h period measured milk production (29 out of 32 studies), whereas only 15 of the 62 studies collecting HM samples at one time point and 2 of the 7 studies that collected HM samples via other methods had assessed milk production as well as milk composition.

Determinants of methods of collection

A broad range of collection methods were used, independent of the macronutrient(s) being measured in the study (**Figure 4**). Most studies collected samples at one time point, regardless of whether they reported fat concentration (50 of 85 studies), protein concentration (42 of 70 studies), or lactose concentrations (35 of 54 studies). For each macronutrient, the method of collection used was highly variable (Figure 4).

Geographically, 31 studies were performed in United States, with 24 collecting full expression (15 at one time point and 9 across 24 h), whereas of the 11 studies conducted in Australia, 7 adopted the collection of 24 h pre- and post-feed samples. The 2 largest studies (n = 155 and 156 participants) both utilized full breast expression collected at one time point. No other clear patterns as to the choice of method in the individual studies could be identified.

Determinants of macronutrient concentrations

The mean fat, protein, and lactose concentrations in colostrum, transitional, and mature HM obtained in samples collected from mothers of term infants using different HM collection methods are presented in **Tables 2–4**. Relatively few studies meeting the inclusion criteria measured macronutrient concentrations in transitional HM or colostrum; however, the available data suggested that fat concentration was lower and protein concentration higher in colostrum and transitional HM compared with mature HM (Tables 2 and 3), while lactose concentration was similar across lactation stages (Table 4). In mature HM, fat and protein concentrations were similar between studies that collected full expressions across 24 h or pooled pre- and post-feed samples either at one time point (morning) or across 24 h, but were higher in studies that collected either post-feed samples or a full expression samples at one time point (morning) (Tables 2 and 3). In the case of fat, but not protein, the concentrations in HM were lower in studies that collected pre-feed samples (Table 2). Lactose concentration of HM was similar in studies

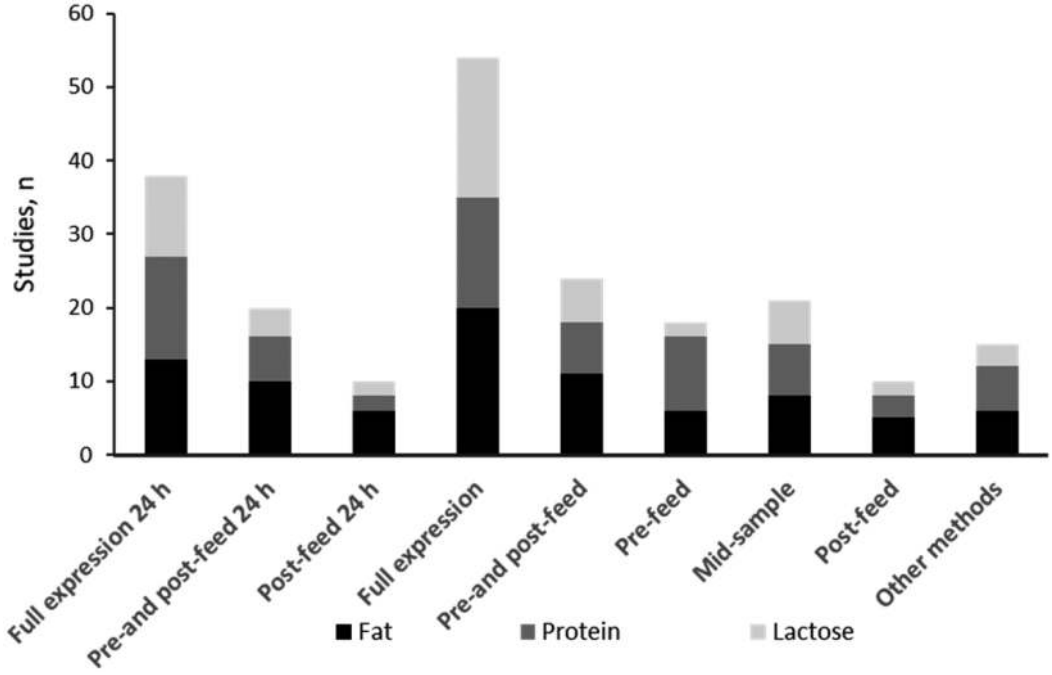

FIGURE 4 The number of studies applying each of the different human milk collection methods for the measurement of fat, protein, and lactose.

that had undertaken a full expression either at one time point or across 24 h and for pre- and post-feed or post-feed across a 24 h period, and there were insufficient studies to compare between other methods (Table 4). A full list of macronutrient concentrations obtained for all studies included in the review, including studies undertaken in preterm infants and studies where the stage of lactation was not stated or unclear and therefore not included in the major synthesis, is presented in **Supplemental Tables 3–5**.

Comparison of analytical methods

Sixteen different analytical methods were reported for fat analysis, with the most common being the creamatocrit method (n = 28) and Folch extraction (n = 13). While direct comparisons between studies are complicated by the significant variability in HM fat concentrations existing between individual women and between populations, there did not appear to be any significant difference in the average fat concentration of HM at any stage of lactation depending on the analytical method used. It is important to note, however, that not all studies applied the same formula for converting the creamatocrit value to the percentage fat, making it difficult to undertake a direct comparison.

Protein was analyzed by 16 different analytical methods across the studies, with the most popular being the Kjeldahl method, including micro-Kjeldahl and modified versions (n = 22) and Lowry assay (and modifications thereof) (n = 7). Protein concentrations varied considerably between studies, independently of the analytical and collection methods, but no clear patterns could be identified. Of the studies that reported protein concentrations, 9 reported concentrations of protein nitrogen (36, 41, 45, 61, 116) and total nitrogen (34, 35, 52, 74).

A total of 15 different analytical methods were used for the measurement of lactose concentrations, with the most common being the enzymatic method, including enzymatic-spectrophotometer (n = 14), followed by automated analyzer (n = 10; Yellow Springs Instrument and Technicon). The choice of analytical method seemed to be a more important factor influencing lactose concentration than method and time of milk collection. For instance, some studies using the Mid-Infrared Milk (MIRIS) analyzer, Sweden (n = 5) tended to report lower

TABLE 2 Fat concentrations in colostrum, transitional, and mature milk according to human milk collection method[1]

		Mature		Transitional		Colostrum	
Method of collection	Time of collection	Included studies, n (total participants, n)	Milk concentration, g/L	Included studies, n (total participants, n)	Milk concentration, g/L	Included studies, n (total participants, n)	Milk concentration, g/L
Full expression	24 h period	8 (424)	35.2 ± 3.1	1 (23)	31.0 ± 10.0	1 (23)	18.0 ± 6.0
Pre- and post-feed	24 h period	6 (148)	34.5 ± 10.1	0	—	0	—
Post-feed	24 h period	5 (332)	44.6 ± 2.5	0	—	4 (220)	17.6 ± 4.1
Full expression	Morning	6 (449)	42.4 ± 13.1	2 (77)	36.5 ± 1.9	1 (137)	24.2 ± 1.0
Pre- and post-feed	Morning	4 (180)	36.0 ± 11.5	0	—	0	—
Pre-feed	Morning	2 (114)	31.1 ± 5.3	0	—	0	—
Mid-feed	Morning	1 (103)	38.9 ± 6.5	0	—	0	—
Post-feed	Morning	1 (43)	30.6 ± 6.5	1 (43)	31.1 ± 15.3	2 (179)	28.7 ± 3.8

[1] Data are presented as means ± SDs unless otherwise indicated.

TABLE 3 Protein concentrations in colostrum, transitional, and mature milk according to human milk collection method[1]

Method of collection	Time of collection	Mature		Transitional		Colostrum	
		Included studies, *n* (total participants, *n*)	Milk concentration, g/L	Included studies, *n* (total participants, *n*)	Milk concentration, g/L	Included studies, *n* (total participants, *n*)	Milk concentration, g/L
Full expression	24 h period	8 (432)	9.4 ± 2.1	1 (23)	17.0 ± 2.0	1 (23)	23.0 ± 6.0
Pre- and post-feed	24 h period	4 (52)	9.9 ± 1.6	0	—	0	—
Post-feed	24 h period	1 (112)	16.0	0	—	0	—
Full expression	Morning	4 (233)	13.3 ± 4	2 (77)	11.3 ± 1.3	1 (137)	29.0 ± 2.2
Pre- and post-feed	Morning	4 (207)	10.4 ± 1.8	1 (91)	13.3 ± 3.4	1 (91)	18.0 ± 3.5
Pre-feed	Morning	2 (101)	12.6 ± 0.9	0	—	2 (110)	18.9 ± 0.1
Mid-feed	Morning	1 (103)	16.1 ± 1.6	0	—	0	—
Post-feed	Morning	0	—	0	—	0	—

[1] Data are presented as means ± SDs unless otherwise indicated.

concentrations (72, 108) compared with studies using other methods.

Direct comparison of methods of collection within a study

A total of 21 studies included in this systematic review directly compared the same analyte (fat, $n = 10$; protein, $n = 7$; lactose, $n = 4$) for HM samples obtained using different collection methods. All studies reported that fat concentration differed according to the method of collection, with post-feed concentrations significantly higher compared with pre-feed concentrations. There were, however, no systematic differences reported for either protein or lactose concentrations according to the method of collection.

Quality assessment

The risk of bias assessment is summarized in Table 5. Overall quality of the studies was relatively low, with a median ± SD score of 5.6 ± 1.6 (out of a possible 8.5) across all studies, and 29 studies scoring <50% of the maximum score. The highest scores were similar for studies that utilized full expression across 24 h (5.9 ± 1.7), mid-feed (5.9 ± 1.1), post-feed across 24 h (5.8 ± 1.2), and pre- and post-feed (5.8 ± 1.4) across 24 h, while those studies in which HM samples were collected post-feed at one time point appeared to have lower scores (3.9 ± 2.2).

A large number of studies, particularly those that collected samples at only one time point, failed to report key pieces of information. Of relevant studies collecting samples at one time point (full expression, pre- and post-feed, pre-feed, and most of the other methods), less than half of the studies standardized the time since last feed or expression for which the sample was collected ($n = 22$ of 55 studies). The majority of samples utilizing Bradford assays for protein measurement ($n = 9$ of 12 studies) used HM samples as standards.

Discussion

The current review describes the first systematic evaluation of the range of sampling methodologies used in studies reporting HM composition. The review has highlighted the wide range of collection methods applied in the field, as well as the substantial variability between studies in other factors that may impact HM composition, including stage of lactation, whether infants are term or preterm, and the analytical method applied to measure macronutrient concentrations. This information provides an important basis for developing recommendations for best practices for HM collection for compositional analysis, which, if it were to be adopted by researchers in the field, would considerably expand the opportunities for combining data from different research groups and thus advance knowledge in the field.

Selecting a HM sampling method requires researchers to identify the most representative method of collection that is feasible for a given study. Based on the evidence synthesized in this review, and the relative strengths and weaknesses of different collection methods (Table 6), collection of pre- and post-feed samples (and pooling prior to analysis) for all feeds across a 24 h period represents the most appropriate alternative to the "gold standard." Pre- and post-feed sampling avoids issues associated with collection of full breast expressions, while still obtaining representative compositional data and accounting for diurnal variations in HM composition. Where collection of samples at all feeds across 24 h is not feasible,

TABLE 4 Lactose concentrations in colostrum, transitional, and mature milk according to human milk collection method[1]

Method of collection	Time of collection	Mature		Transitional		Colostrum	
		Included studies, *n* (total participants, *n*)	Milk concentration, g/L	Included studies, *n* (total participants, *n*)	Milk concentration, g/L	Included studies, *n* (total participants, *n*)	Milk concentration, g/L
Full expression	24 h period	6 (224)	63.5 ± 6.3	1 (23)	67.0 ± 5.0	1 (23)	62.0 ± 9.0
Pre- and post-feed	24 h period	3 (51)	63.2 ± 2.8	0	—	0	—
Post-feed	24 h period	1 (112)	62.0 ± 9	0	—	0	—
Full expression	Morning	4 (237)	64.3 ± 8.6	1 (56)	65.0 ± 9.0	2 (177)	57.9 ± 5.9
Pre- and post-feed	Morning	3 (116)	71.8 ± 5.4	—	—	0	—
Pre-feed	Morning	1 (51)	69.5 ± 4.9	—	—	1 (51)	69.0
Mid-feed	Morning	0	—	—	—	0	—
Post-feed	Morning	0	—	—	—	0	—

[1] Data are presented as means ± SDs unless otherwise indicated.

TABLE 5 Risk of bias assessment for studies measuring macronutrient concentrations in human milk[1]

Reference	Representativeness of cohort	Controls for confounders	Sample size (small, medium, or large study)	State mode of feeding	Standard postpartum HM collection	State mode collection (manual/pump)	State gestational age	Report analytical method	Score
Full expression across 24 h									
Anderson et al., 1983 (30)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Beijers et al., 1992 (31)	✓	x	Small	x	x	✓	✓	✓	4.5
Bishara et al., 2008 (32)	✓	x	Small	x	✓	✓	✓	✓	5.5
Boutte et al., 1985 (33)	✓	x	Small	✓	x	✓	x	✓	4.5
Brown et al., 1982 (34)	x	x	Small	x	x	✓	x	✓	2.5
Brown et al., 1986 (35)	x	x	Medium	✓	✓	✓	x	✓	5
Butte et al., 1984 (36)	✓	x	Small	✓	✓	✓	✓	✓	6.5
Cregan et al., 2002 (37)	✓	✓	Small	✓	✓	x	✓	✓	6.5
Heinig et al., 1993 (38)	✓	✓	Medium	✓	✓	x	✓	✓	7
Lovelady et al., 1990 (39)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
McCrory et al., 1999 (40)	x	✓	Medium	✓	✓	✓	✓	✓	7
Motil et al., 1997 (41)	✓	✓	Small	✓	✓	x	✓	✓	6.5
Perez-Escamilla et al., 1995 (42)	✓	✓	Large	✓	✓	✓	✓	✓	8.5
Stafford et al., 1994 (43)	✓	x	Small	x	x	✓	x	✓	3.5
Stellwagen et al., 2013 (44)	x	x	Small	✓	x	✓	✓	✓	4.5
Stuff et al., 1989 (45)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Overall									5.9 ± 1.7
Pre- and post-feed across 24 h									
Cannon et al., 2015 (9)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Daly et al., 1993 (46)	x	x	Small	✓	✓	x	x	✓	3.5
Jackson et al., 1988 (47)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Kent et al., 2006 (10)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Khan et al., 2013 (6)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Mitoulas et al, 2002 (48)	✓	x	Small	✓	✓	✓	✓	✓	6.5
Perrella and Geddes, 2016 (49)	x	x	Small	✓	✓	✓	✓	✓	5.5
Prentice et al., 1981 (50)	✓	x	Medium	x	x	✓	x	✓	4
Saint et al., 1986 (51)	x	x	Small	✓	x	✓	✓	✓	4.5
Valentine et al., 1994 (52)	x	x	Small	x	✓	✓	x	✓	3.5
Overall									5.8 ± 1.8

(*Continued*)

TABLE 5 (*Continued*)

Reference	Representativeness of cohort	Controls for confounders	Sample size (small, medium, or large study)	State mode of feeding	Standard postpartum HM collection	State mode collection (manual/pump)	State gestational age	Report analytical method	Score
Post-feed across 24 h									
Agostoni et al., 2001 (53)	✓	x	Large	✓	✓	x	✓	✓	6.5
Agostoni et al., 2003 (54)	✓	x	Large	✓	✓	x	✓	✓	6.5
Bauer and Gerss, 2011 (55)	✓	✓	Large	x	✓	✓	✓	✓	7.5
Marangoni et al., 2000 (56)	✓	x	Small	x	✓	x	✓	✓	4.5
Marangoni et al., 2002 (57)	✓	x	Medium	x	✓	x	✓	✓	5
Morton et al., 2012 (58)	x	x	Medium	x	✓	✓	✓	✓	5
Overall									5.8 ± 1.2
Full expression									
Aksit et al., 2002 (59)	✓	x	Medium	✓	✓	✓	✓	✓	7
Barbosa et al., 1997 (60)	x	x	Small	x	✓	✓	x	✓	3.5
Butte et al., 1984 (61)	✓	✓	Small	x	✓	✓	✓	✓	6.5
Butte et al., 1992 (62)	✓	✓	Small	x	✓	✓	✓	✓	6.5
Chen et al., 1998 (63)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
De Pee et al., 1997 (64)	✓	x	Large	x	✓	✓	x	✓	5.5
Dewey and Lönnerdal, 1983 (65)	✓	x	Small	✓	✓	✓	x	✓	5.5
Foda et al., 2004 (66)	x	x	Small	x	✓	✓	x	✓	3.5
Goran et al., 2017 (67)	✓	x	Small	✓	✓	✓	✓	✓	6.5
Heon et al., 2016 (68)	✓	x	Small	✓	✓	✓	✓	✓	6.5
Jackson et al., 1994 (69)	✓	x	Medium	x	✓	✓	✓	✓	6
Lönnerdal et al., 1976 (27)	✓	x	Large	x	✓	✓	x	✓	5.5
Marquis et al., 2003 (70)	x	✓	Large	x	✓	✓	✓	✓	6.5
Masters et al., 2002 (71)	✓	x	Small	x	x	✓	x	✓	3.5
Moran-Lev et al.,2015 (72)	x	✓	Small	✓	✓	✓	✓	✓	6.5
Mosley et al., 2007 (73)	✓	✓	Small	✓	✓	✓	x	✓	6.5
Neubauer et al., 1993 (74)	✓	✓	Small	x	✓	✓	✓	✓	6.5
Park et al., 1999 (75)	x	x	Small	x	x	✓	x	✓	2.5
Perrin, 2015 (76)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Ritzenthaler et al., 2005 (77)	✓	x	Small	x	x	✓	x	✓	3.5
Sauer et al., 2017 (78)	x	x	Small	x	✓	✓	✓	✓	4.5
Szlagatys-Sidorkiewicz et al., 2013 (79)	✓	x	Large	✓	✓	✓	✓	✓	7.5
Thurl et al., 1993 (80)	x	x	Small	✓	✓	✓	x	✓	4.5
Wack et al., 1997 (81)	✓	x	Large	x	✓	✓	x	✓	5.5
Williams et al., 2017 (82)	✓	x	Small	x	x	✓	x	✓	3.5
Young et al., 2017 (83)	✓	✓	Medium	✓	✓	✓	✓	✓	8
Overall									5.6 ± 1.5

(*Continued*)

TABLE 5 (*Continued*)

Reference	Representativeness of cohort	Controls for confounders	Sample size (small, medium, or large study)	State mode of feeding	Standard postpartum HM collection	State mode collection (manual/pump)	State gestational age	Report analytical method	Score
Pre- and post-feed									
Da Cunha et al., 2005 (84)	✓	✓	Medium	✓	✓	✓	✓	✓	8
Fornes and Dorea, 1995 (85)	✓	x	Small	✓	✓	✓	x	✓	5.5
Gridneva et al., 2017 (86)	✓	✓	Small	x	✓	✓	✓	✓	6.5
Grote et al., 2016 (8)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Hahn et al., 2018 (87)	x	x	Medium	✓	✓	x	x	✓	4
Hassiotou et al., 2013 (88)	✓	✓	Small	x	✓	✓	x	✓	5.5
Karatas et al., 2011 (89)	✓	x	Small	✓	✓	x	✓	✓	5.5
Kugananthan et al., 2017 (7)	✓	✓	Medium	✓	✓	✓	✓	✓	8
McDaniel et al., 1989 (90)	x	✓	Small	x	x	✓	x	✓	3.5
Michaelsen et al., 1994 (91)	✓	x	Medium	✓	x	x	✓	✓	5
Prentice et al., 1981 (92)	✓	✓	Large	x	x	✓	x	✓	5.5
Schueler, 2011 (93)	✓	x	Small	✓	✓	✓	x	✓	5.5
Overall									5.8 ± 1.4
Pre-feed									
Al-Awadi and Srikumar, 2001 (94)	✓	x	Small	✓	✓	x	x	✓	4.5
Antonakou et al., 2013 (95)	✓	x	Medium	✓	✓	✓	✓	✓	7
Bachour et al., 2012 (96)	x	x	Medium	x	x	✓	x	✓	3
Chavalittamrong et al., 1981 (97)	x	x	Large	x	✓	✓	x	✓	4.5
Donangelo et al., 1989 (98)	✓	x	Medium	✓	✓	✓	x	✓	6
Dudzik et al., 2008 (99)	✓	x	Medium	✓	✓	✓	✓	x	6
Fujita et al., 2012 (100)	✓	✓	Medium	✓	x	✓	x	✓	6
Khin-Maung et al., 1980 (101)	x	x	Small	✓	✓	✓	x	✓	4.5
Picciano and Guthrie, 1976 (28)	✓	x	Small	✓	✓	✓	✓	✓	6.5
Trugo et al.,1988 (102)	x	x	Medium	✓	✓	✓	✓	✓	6
Tyson et al., 1976 (29)	✓	x	Small	✓	✓	✓	✓	✓	6.5
Overall									5.5 ± 1.2

(*Continued*)

TABLE 5 (*Continued*)

Reference	Representativeness of cohort	Controls for confounders	Sample size (small, medium, or large study)	State mode of feeding	Standard postpartum HM collection	State mode collection (manual/pump)	State gestational age	Report analytical method	Score
Mid-feed									
Allen et al., 1991 (103)	x	x	Small	✓	✓	✓	x	✓	4.5
Dutta et al., 2014 (104)	✓	x	Small	✓	✓	✓	✓	✓	6.5
Kon et al., 2014 (105)	✓	x	Large	x	✓	✓	✓	x	5.5
Lubetzky et al., 2015 (106)	✓	x	Medium	x	✓	✓	✓	✓	6
Mandel et al., 2005 (107)	✓	✓	Medium	✓	✓	✓	✓	✓	8
Pines et al., 2016 (108)	✓	x	Medium	✓	✓	✓	x	✓	6
Quinn, 2013 (109)	✓	✓	Large	x	x	✓	x	✓	5.5
Quinn et al., 2016 (110)	✓	✓	Medium	x	x	✓	x	✓	5
Overall									5.9 ± 1.1
Post-feed									
Bener et al., 2001 (111)	x	x	Small	x	x	x	x	✓	1.5
Cant et al., 1991 (112)	x	x	Small	x	x	x	x	✓	1.5
Moltó-Puigmartí et al., 2011 (113)	✓	x	Small	x	✓	✓	✓	✓	5.5
Rakicioglu et al., 2006 (114)	✓	x	Small	✓	✓	✓	x	✓	5.5
Vitólo et al., 1993 (115)	x	x	Large	x	✓	✓	✓	✓	5.5
Overall									3.9 ± 2.2
Other methods									
Bassir, 1959 (116)	x	x	Small	x	✓	✓	x	x	2.5
Britton, 1986 (117)	x	x	Small	✓	✓	✓	✓	✓	5.5
De Luca et al., 2016 118)	✓	✓	Large	✓	✓	✓	✓	✓	8.5
Eilers et al., 2011 (119)	✓	x	Medium	x	✓	✓	✓	x	5
Neville et al., 1984 (120)	✓	x	Small	✓	x	✓	✓	✓	5.5
Shehadeh et al., 2006 (121)	✓	x	Medium	✓	✓	✓	✓	✓	7
Woolridge et al., 1990 (122)	✓	x	Small	x	✓	x	✓	✓	4.5
Overall									5.5 ± 1.9

[1]The quality-assessment scores for each collection method are presented as means ± SDs. Sample size is defined as small = studies with <50 participants, medium = studies with between 50 and 100 participants, and large = studies with >100 participants. The checkmark symbol indicates yes and cross symbol indicates no.

TABLE 6 Methodological and practical considerations of different human milk collection methods[1]

Time of collection	Methods of collection	Best represents feed	Accounts for diurnal changes	Minimal interference with infant feeding	Suitable for large studies	Minimal issues HM limited supply	Minimal issues transport/storage of samples	Less burden mothers/infants
24 h sampling	Full expression	✓	✓	x	x	x	x	x
24 h sampling	Pre- and post-feed	x	✓	✓	x	x	x	x
24 h sampling	Post-feed	x	✓	✓	x	✓	x	x
One sample	Full expression	✓	x	x	x	x	✓	x
One sample	Pre- and post-feed	x	x	✓	✓	✓	✓	✓
One sample	Pre-feed	x	x	✓	✓	✓	✓	✓
One sample	Mid-feed	x	x	✓	✓	✓	✓	✓
One sample	Post-feed	x	x	✓	✓	✓	✓	✓

[1]HM, human milk. The checkmark symbol indicates yes and cross symbol indicates no.

collection of pre- and post-feed samples 3 times/d may be more practical and would still account for diurnal variations in composition. If sample collection at one time point is the only pragmatic option, for example in large population-based studies, the recommended approach would be collection of pre- and post-feed samples (pooled for analysis) at the same time of day for all participants, preferably the morning, and at a consistent time after the previous feeding (at least 2 h when the breast has synthesized a reasonable amount of milk). These recommendations are particularly relevant for the analysis of fat concentration, given that the amount of milk in the breast is related to fat concentration and this varies over the day (10). In addition, other factors, such as the breast from which samples are collected, how milk samples are obtained (hand expression vs. manual/electric pump), as well as stage of lactation should also be standardized across all participants. Further to this reporting, and ideally standardizing, other factors known to influence HM composition [including mode of feeding (exclusively breastfeeding, partial breastfeeding, and formula feeding), term vs. preterm, stage of lactation] are also critical (7, 8, 55, 123–125). The key points to consider when planning a study measuring HM composition are presented in **Table 7**.

This systematic review has identified a number of different HM collection methods that were applied across the studies. While, perhaps unsurprisingly, the method of collection used tended to be consistent across research groups, there was no clear rationale for selection of the collection method. It was notable, however, that the "gold standard" method of 24 h collection of full expression of all feeds tended to be applied to a greater extent in older studies, and 2 of these studies stated that this method was selected as it was believed to be the most representative of milk consumed by the infant (30, 39). More recent studies, however, tended to collect one expression or pre-/post-feed samples at one time point across the day, usually the morning. This may reflect the tightening of ethical requirements around human research over time, particularly the need to justify the burden to participants in the context of the research question. In addition, full expressions require the infant to be fed via other means (bottle or cup), which then limits the population for recruitment. Collection of full breast expressions from all feedings over a 24 h period represents a substantial burden for the mother, is not practical for large population-based studies, and may have the potential to interfere with normal breastfeeding (126, 127). Indeed, 2 of the studies in this review elected to collect samples at only one time point to minimize interference with infant feeding (103, 110). Consequently, it can be argued that applying the "gold standard" approach may restrict the women who are willing and able to participate, and that the results obtained from these studies may therefore not be representative of the general population of breastfeeding women.

In the move away from collection of full expressions at all feeds across a 24 h period, towards collection at one time point, there also appeared to be parallel tendency to collect smaller sample volumes either pre-, post-, or during a feed. While assessing the composition of a full expression is generally thought to be most representative of the milk that the infant consumes (36, 127), 1 study included in this review directly compared a full expression with pooled pre- and post-feed samples from the same woman and found little difference in protein concentrations between these approaches (32). This is important, since full breast expressions have a higher participant burden than spot collections and may not be possible in certain women/population groups. If equivalent results can be obtained by pooling pre- and post-feed samples, this increases the opportunity to access broader population groups and undertake larger studies, both of which are important for the generalizability of the findings.

We had expected that the macronutrient measured would be an important determinant of the choice of method of HM

TABLE 7 Key recommendations to consider when planning a study measuring human milk macronutrient composition[1]

Overall	Report and standardize			
	Collection procedure	Other factors	Analytical method	Milk production
Same sampling procedure for all participating women	Time of day of collection Collection mode (hand expression and/or breast pump) Collection breast Time since last feed or expression	Stage of lactation Infant gestational age Mode of feeding (exclusively or partial breastfeeding)	Validated for HM Use of HM standards, where applicable	Maternal milk production and/or Infant milk intake across 24 h whenever possible

[1]HM, human milk.

collection, particularly for fat concentration (10). However, this speculation was not supported by the findings. Nevertheless, the collection method did influence macronutrient composition. Higher fat concentrations in post-feed samples compared with pre-feed samples were expected, given emptying of the breast has been consistently associated with increases in fat concentration across a feed or breast expression (9, 10). However, the higher mean HM protein concentration reported by studies that had collected samples post-feed or full expression at one time point was not anticipated (6). This may imply that protein concentrations in HM have the potential to be impacted by collection method, although other factors, including whether mothers were partially or full breastfeeding and the analytical method applied, may also contribute to these differences. Indeed, synthesizing the information related to average macronutrient composition by lactation stage and collection method was complicated by the considerable heterogeneity between studies in relation to other factors known to impact HM composition (7, 8, 55, 123–125), which again emphasizes the importance of reporting, and ideally standardizing, these factors.

An unexpected finding was the substantial variability in the analytical methods that were applied to assess all macronutrients in the HM samples, which also complicated comparisons between studies and needs to be considered when interpreting the results. There was an overall lack of detail in reporting of analytical methods, as well as handling of the samples post-collection. As sample treatment and management, including transport conditions, homogenization, freeze-thaw cycles, and duration and conditions of storage, all have the potential to impact HM composition (128–130), it is critical that these factors are reported with sufficient detail to enable results of compositional analyses to be interpreted appropriately.

Our review highlights the general low quality of many of the included studies in the HM area, particularly regarding lack of detailed information on HM collection procedures. Many studies were excluded at the full-text screening stage due to not reporting key factors, including time of day of collection or method of sample collection. Most studies did not provide detailed information on other factors that could impact HM composition, including stage of lactation, parity, and gestational age of the infant at birth. Time since the last breastfeeding that HM samples were collected was also reported in less than half of the studies collecting samples at one time point, despite evidence that HM samples collected at least 2 h after a previous feeding (131), and when the breast is full or near full, provide the most reliable representation of the HM composition of the subsequent feed (132). Most included studies did not assess measures of maternal HM production or infant intake, limiting the ability to account for volume, which could impact sampling for fat concentration. Inclusion of objective measures of milk production/infant intake, such as test weighing or deuterium-labeled water, in addition to compositional analysis, would improve estimates of variability due to maternal and infant factors.

A major strength of this systematic review is the comprehensive analysis across broad range of studies, geographical regions, study populations, and study designs, including observational cohort (longitudinal and cross-sectional), interventional, and case studies. Further, lactating women across different regions, populations, settings (urban and rural), ethnic and sociodemographic backgrounds, and stages of lactation were included. A clear limitation of our review was the restricted number of studies in which we could undertake comparison of results from different studies using the "gold standard" sampling method versus alternative methods of collection and to calculate the average concentrations at different lactation stages, which limited the generalizability of these findings.

In conclusion, this systematic evaluation highlights the wide range of sampling methods applied in studies assessing HM composition around the world. Our findings, particularly the quality assessment, reinforce the need for establishing a standardized method for HM collection to ensure accurate analysis of milk components with respect to infant outcomes. In addition, the reporting and standardization of collection procedures, validation of analytical methodology, and inclusion of HM production measures would contribute to a more representative compositional analysis. Our work provides research groups in this field with valuable guidance on the details required when designing and reporting studies, particularly in relation to the methodology used for HM collection.

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