RESEARCH PAPER

Rapid profiling of triglycerides in human breast milk using LESA-FTMS reveals new VLCFAs and differences within individuals

Albert Koulman[1-3,], Samuel Furse[3,4], Mark Baumert[5], Gail Goldberg[6,7], Les Bluck[6,†].*

[1] Cambridge Lipidomics Biomarker Research Initiative, Elsie Widdowson Laboratory, MRC HNR, Cambridge, CB1 9NL UK,
[2] Lipid Profiling Signalling group, MRC HNR, Cambridge, CB1 9NL United Kingdom.
[3] Core Metabolomics and Lipidomics Laboratory, MRL, Institute of Metabolic Science, Level 4, Pathology Building, Addenbrooke's Hospital, Cambridge CB2 0QQ, United Kingdom.
[4] Department of Biochemistry, University of Cambridge, Hopkins Building, Tennis Court Road, Cambridge, CB2 1QW, United Kingdom.
[5] Advion Ltd, Kao Hockham House, Edinburgh way, Harlow, Essex, CM20 2NQ, United Kingdom.
[6] Nutrition and Bone Health group MRC HNR, Cambridge, CB1 9NL United Kingdom.
[7]MRC Keneba, The Gambia Physiological Modelling of Metabolic Risk MRC HNR, Cambridge, CB1 9NL
**Correspondence should be addressed to: ak675@medschl.cam.ac.uk*
[†]Deceased.

TITLE RUNNING HEAD: Human breast milk differs within individuals and contains VLCFAs.

KEYWORDS: Lipid profile, lipidomics, infant nutrition, milk, dried milk spots, very long chain fatty acids.

Abstract

Rationale

In this paper, we describe a novel method for preparing milk samples and profiling the triglyceride fraction from them. This method was used to explore how the triglyceride (TG) profile of milk modulates as lactation progresses and how the TG profile differs between breasts.

Methods

Fresh milk was spotted onto Whatman filter paper and air-dried. Liquid Extraction Surface Analysis, coupled to Fourier Transform Mass Spectrometry (LESA-MS) was adapted for molecular profiling. Collision-Induced Dissociation (CID) was used to profile fatty acid residues.

Results

LESA-MS produced the relative abundance of all isobaric TGs described and showed that mammary glands within one individual can produce a different profile of TGs. CID was used to uncover the configuration of isobaric triglycerides, indicating the relative amounts of the fatty acids contributing to that triglyceride's mass. This also indicated the presence of very long chain fatty acids (C26:0 and C26:1) that have not been reported before in human breast milk.

Conclusions

We conclude that spotting on paper and the use of LESA-MS and CID on milk spots is not only a means for analysing milk in unprecedented detail for this preparation time, but is also amenable to conditions in which collecting and storing fresh milk samples for detailed profiling is prohibitively difficult.

Introduction

There is an increasing body of evidence that suggests that breast milk is important not only for the short-term health and development of infants[1], but also for longer term health, cognition and reduction of risk of disease[2-4]. To obtain a better understanding of how breast milk affects infants' health, as well as how the maternal diet and lifestyle affect breast milk composition, it is necessary to be able to measure the composition of human breast milk in detail, including its richest source of energy, triglycerides (TGs).

TGs are at least 50× as abundant as phospholipids in milk[5, 6]. There are many studies on the total quantity of triglycerides in milk[7-9], but molecular profiling has thus far typically be restricted to gross profiles of the fatty acid (FA) composition. Thus, detailed information about the relative abundance of individual isoforms of TGs is lacking, as is an understanding of the kinetics of their production during human lactation. This paucity of data can be ascribed to a lack of specific and adequate methods for profiling triglycerides that are compatible with the need for running larger numbers of samples.

The gross FA profile of a biological sample typically involves preparation of Fatty Acid Methyl Esters (FAMEs) that are then profiled by gas chromatography (GC)[7]. This approach has a long history, including in studies of milk[10]. This commonality facilitates the interpretation and comparison of results between studies. However, when the TG fraction is hydrolysed, information about the combinations of the acids in TGs and their positions on the glyceryl moiety is lost. Furthermore, the long sample preparation time required for this method invites variability and prohibits the profiling of larger sample numbers and thus bigger cohorts. Typical analysis times using this method are 30-60 min per sample.

Methods developed more recently have yet to gain significant traction in studies on milk. The analysis of triglycerides using HPLC is time consuming and lacks resolution[11]. The combination of preparative HPLC or TLC to separate the triglycerides, hydrolysis, preparation and analysis of the methyl esters of constituent fatty acids by gas chromatography is detailed but only rarely performed[12] as it too is time consuming and not really compatible with larger sample numbers. Very recently, LC-MS/MS has been used to profile the triglycerides fraction of human milk[13-16], however it suffers from poor fractionation of triglycerides by reverse phase chromatography, and the solvents necessary for normal phase chromatography reduce the already poor ionisation efficiency of triglycerides[17].

Paper chromatography was used for several decades because of its simplicity and effectiveness, but in recent years its use has declined due to lack of resolution and poor compatibility with sensitive detection methods. However, paper is an attractive medium for short-term sample storage as it is amenable to anaerobic storage at low temperatures and does not require anti-coagulants. Second, it requires only a small sample volume (10-50 µL or in other words, just one drop) and little subsequent sample preparation[18]. This contrasts with the collection of fresh milk samples that are required for LC-MS/MS and other methods, and are typically considerably larger and more difficult to store than dried milk spots.

Luckily, the introduction of ambient ionisation methods such as Desorption Electrospray Ionisation (DESI) and Direct Analysis in Real Time (DART) have made it possible to revisit the use of paper for sample spotting and make it compatible with state-of-the-art mass spectrometry. Furthermore, recent work with blood samples has shown that blood spots on filter paper can be used to obtain a representative lipid profile when compared to fresh blood or plasma[15, 16]. In that work, it was necessary to make adjustments for the presence of artefacts (*e.g.* oxidised lipids)[19], which may also appear in biological samples such as milk. However, sample preparation and running times were not excessive.

The need for convenient storage of milk samples and for processing larger numbers of samples, and the suggestion that paper may be a suitable medium, led us to the hypothesis is that filter paper is a suitable matrix for milk samples. Further, we suggest that it will absorb proteins and salts, the latter potentially interfering with lipid ionisation, leaving fat globules on the surface of the paper in a manner for analysis by ambient ionisation techniques such as Liquid Extraction Surface Analysis (LESA). We tested the hypothesis using a LESA coupled to a high resolution mass spectrometer (for example a FTMS), in order to give a detailed triglyceride profile of milk fat. Established multivariate data analyses were used to explore the data[21]. Qualitative differences in isomeric triglycerides were determined using a linear ion trap[22].

In this proof-of-principle research paper, we demonstrate how LESA-FTMS can be used for the relative quantification of the triglycerides in breast milk. We developed the method and tested it on human breast milk from a small cohort of women from The Gambia, and compared the relative triglyceride profiles to values reported already.

Experimental Methods

Chemicals— Solvents were purchased from *Sigma-Aldrich Ltd* (Gillingham, Dorset, UK) of at least HPLC grade and were not purified further. Lipid standards were purchased from *Avanti Polar lipids* (Alabaster, AL; *via* Instruchemie, Delfzijl, NL) and used without purification. Consumables were purchased from *Sarstedt AG & Co* (Leicester, UK).

Lipid standards—In this study we used 0.6 μM 1,2-di-O-octadecyl-sn-glycero-3-phosphocholine, 1.2 μM 1,2-di-O-phytanyl-sn-glycero-3-phosphoethanolamine, 0.6 μM C8-ceramide, 0.6 μM N-heptadecanoyl-D-erythro-sphingosylphosporylcholine, 6.2 μM undecanoic acid, 0.6 μM trilaurin.

Ethics— All procedures were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All mothers gave informed written consent after an oral explanation in the local language. The study was approved by the MRC/Gambian Government Ethics Committee.

Sample preparation—The milk was thawed, agitated and spotted on Whatman filter paper (cat. No 1001 055) and air-dried under a gentle flow of N_2. Samples were used immediately.

Mass spectrometer—All samples were analysed using a Traversa NanoMate system (*AdvionBioSciences*, Inc. Ithaca, NY, USA) coupled to either Exactive or to an LTQ velos Orbitrap mass spectrometer (both instruments from *Thermo Scientific*, Hemel Hempstead, UK). A nanoelectrospray voltage of 1·55 kV and gas pressure of 0·2 psi was applied in all experiments. Customised robotic arm movements and custom liquid handling for a surface analysis was set up in the AUI panel of the ChipSoftManager software controlling the NanoMate. The Exactive was set up to collect data in positive mode with 1 Hz scan rate for maximum resolution. The LTQ Velos Orbitrap was set to collect data in positive mode with the following 5 scan events (SE):

SE1:- FTMS 300-2000 *m/z* (res = 100000);

SE2:- data dependent ITMS MS^2 of most intense ion from parent list (fragmentation settings: Activation type = CID; normalized collision energy = 35%; isolation width = ± 1 *m/z*; activation Q = 0·25; activation time = 30 ms; minimal intensity = 500);

SE3:- data dependent IMTS MS^3 most intense ion from MS^2 (same setting as SE 2, except isolation width = ± 2·5 *m/z*);

SE4:- data dependent ITMS MS^3 of 2nd most intense ion from MS2 (same setting as SE 3);
SE5:- data dependent IMTS MS^3 of 3rd most intense ion from MS2 (same setting as SE 3).
The Parent list comprised the ammonium adducts of all possible triglycerides lipids (See *Table S1* for complete list up to ca. 70 masses could be fragmented per sample analysis).

Experimental plan—Mothers in rural areas of The Gambia typically introduce complementary foods after about 4 months and continue to breast feed for 18-24 months. The breast milk samples used in the analyses described in this paper were serendipitous collections from a longitudinal study of maternal calcium ion supplementation conducted in the villages of Keneba and Manduar in the West Kiang region of The Gambia[23]. Only samples from women who were followed up were included in the final analyses. The samples were collected at 2, 13 and 52 weeks lactation from each woman and from each breast. Samples were frozen immediately after collection and stored at -20 °C at MRC Keneba. They were shipped frozen to the UK on dry ice and stored at -80 °C until analysis.

Results

Spotting and sample preparation—Milk was spotted on paper with careful drying by gaseous nitrogen in order to obtain small and compact spots. This is possible by hand-spotting, though reproducibility was better when using automated spotting or larger sample volumes. When the droplet of the extraction solvent did not make proper contact with the spot in the middle, or when the fat layer was compromised, triglycerides appeared as only minor constituents and spectra were inconsistent. During development, we therefore spotted each sample nine times and discarded all analyses that showed poor extraction. Good spotting appears to be important for the success of this method as the quality of LESA is dependent on the formation of an intact hydrophobic layer of fat globules on the paper in combination with the extraction solvent making contact with the fat layer in the middle of the spot to prevent capillary forces of the paper draining the extraction solvent.

We tested a range of solvents for preparing dried milk spots on paper and found that methanol was optimum, with sufficient extraction efficiency but without the problem of the extraction solvent dispersing into the paper. We doped the methanol with ammonium acetate (20 μM) to provide enough ammonium to facilitate the ionisation of the (uncharged) triglycerides. The extraction efficiency of the lipids from the surface of the paper in LESA[24] is dependent on the physico-chemical properties of the surface as well as the properties of the liquid used for the extraction. The analytes need to diffuse rapidly into the extraction solvent, but the surface tension of the droplet of the extraction fluid should not be broken by the surface analysed, because that impairs an effective aspiration of the droplet after extraction.

Data acquisition—The full mass spectrum for each sample, averaged over 10 scans, was exported from the Xcalibur software into MS Excel and searched for the intensities of all the theoretical triglycerides (*Table S1*). These peak areas were then averaged over 3 repeat analyses of the same sample in order to obtain the final value presented here.

Triglyceride profile from individuals—Mass spectra obtained from milk samples prepared and analysed in this way (spotting/LESA-MS) are shown in *Fig. 1*. The samples from each individual comprise milk collected from both breasts. Samples were profiled within about 48h; storage of milk spots for prolonged periods (>14d) at room temperature led to sample degradation. The latter is ascribed to oxidation of the olefin bonds. Additionally, we show a subsection of the mass spectrum of a sample spotted on the same day and either profiled immediately or after storage for 14 days at room temperature in the dark. There was a clear increase in the abundance of oxidised triglycerides

in samples analysed after two weeks of storage at room temperature and in partially aerobic conditions. However, there was no measurable effect within 48 hours. The extent of oxidation was limited to 2-3 % of the original peak and without any distinct effect on the overall triglyceride profile. Oxidation could be slowed down considerably by storing the dried milk spots in a freezer and in under an inert atmosphere.

Triglyceride profile through time—In order to determine the global changes in lipid profile over 50 weeks of lactation, the data from the two breasts were averaged and used in a multivariate analysis to determine underlying patterns of correlation (see *Fig. 2*). For this approach, only data points that showed high reproducibility across all samples were used. The multi-variate data analysis revealed clear positive correlations between the number of carbons and number of double bonds, and number of weeks of lactation.

Identifying isobaric TGs—A number of isoforms of TGs are difficult to identify as they are isobaric to others, *e.g.* the ammonium adduct of TG(36:2) will give a peak at 652.551 *m/z* but the configuration could be 18:1/14:1/4:0, 18:2/14:0/4:0, 18:2/12:0/6:0 amongst others. The intensity of this peak is therefore the sum of the intensity of ions that all have the same molecular formula. Collision-induced dissociation (CID) was used to identify the configuration of isobaric TGs, revealing the identities of the three fatty acid residues (FARs). MS/MS spectra of fragmentation to diglycerides of ammoniated ions of TG(42:0) and TG(42:1) are shown in *Fig. 3*. The loss of fatty acid residues from the triglycerides is shown, providing an insight into the relative distribution of the fatty acids in that particular group of isobaric triglycerides. For TG(42:0) the most abundant loss was C14:0, while for TG(42:1) it was C18:1. This suggests that unsaturation is chiefly invested in the C18:1 FAR and only about 30% is C16:1, with less than 10% being C14:1. The presence of C18:1 correlates with that of shorter chain fatty acids such as C10:0, whose abundance was at least twice as high in TG(42:1) than in TG(42:0). Unfortunately, this method is similar to other mass spectrometry approaches in that it is not possible to identify the position of FARs on the glyceryl moiety (*sn*-1, 3 or 2), or the position or geometry of the double bonds.

Very long chain fatty acids—Despite the limits of MS in determining the regiochemistry of FARs in TGs, the results obtained using this approach have provided novel insights. As well as evidence for short- and medium-length carbon chains (around C10:0 and C16-18 respectively), samples profiled using LESA-MS also indicate the presence of longer FARs. Our results are consistent with the several reports of C24:0 and C24:1 FARs in the triglycerides of human milk [21-23], but the MS^2 spectra of TG(52:0) and TG(54:0) in samples taken a fortnight *post partum* showed losses corresponding to C26:0 FAs. Similarly, TG(52:1) and TG(54:1) showed losses corresponding to C26:1 (*Fig. 4*). The presence of FAs with such long carbon chains has been reported in bovine milk[24, 25], but we could not find any reports in the literature of C26:0 and C26:1 human milk. This result hints that VLFARs may be a feature of milk from all mammals and raises the question of their role *in vivo*.

Variation in the TG profile within individuals—We also used this pilot study to follow up published studies that indicate that provision of milk differs between breasts. Differences between breasts during the same lactation have been reported for volume of milk produced[26, 27] and amount of fat[28, 29], however the triglyceride profile has not yet been reported. LESA-MS was used to profile the TG fraction in milk from two different breasts during the same lactation and the results are shown in *Fig. 5*.

In this example, the difference between breasts was clear for all aspects of the triglyceride profile. One breast gave a profile that was high in lower molecular weight triglycerides while the milk of the other contained a higher level of triglycerides with a higher molecular weight. For example, the MS^2 spectrum of TG(42:1) in the milk from one breast shows a greater loss of C12:0 (*Fig. S1*), but a lower loss of C16:1, than in the other breast. There are also other, subtler differences. Spectra of TG(42:0) indicate that the abundance of TG(18:1/12:0/12:0) is more common than TG(18:1/14:0/10:0) in one breast, while in the ratio is more similar in the other.

Further work is required to understand why different mammary glands in the same woman should produce milk with different TG profile, or how this is controlled. However, spotting-LESA-MS may be amenable to research in this area. The spectra consist mainly of ammonium adducts of triglycerides, but diglycerides (as ammoniated ions), cholesterol lipids (as ammoniated ions) and phospholipids (as protonated ions) are also present. Furthermore, the results obtained by the LESA-MS method are consistent with reports that the relative abundance of phospholipids is ~2%[7, 23].

DISCUSSION

This study was undertaken in order to develop a method to profile milk that had been dried onto paper. The latter was intended as a means for investigating milk production in greater detail, for example in handling larger numbers of samples and from regions where collecting and storing large numbers of samples of fresh milk is impractical. The profiling results suggest that it is possible to carry milk samples on paper. The latter is reliant upon a consistent spotting technique and does not extend the shelf life of the samples at room temperature, however.

Molecular profiling of the samples from a small cohort from The Gambia is consistent with most of our current understanding of fat production in human breast milk. A well-discussed problem in milk analysis is the homogeneity of the sample and how representative this sample is. The changes in composition of milk from a high concentration to a more aqueous composition during a single feed have been observed several times[5, 34]. Similarly, significant differences in the milk composition produced by the two breasts at the same stage of lactation have been reported[26, 27]. Purifying these changes in detail demands great care with sample collection, storing and aliquotting. Understanding the dynamics of the lipid composition and biological factors that drive these have been difficult to study as they demand fast sampling rates and analysis methods with high throughput capabilities. The LESA-MS method described in this paper opens up the possibility of studying fat production in much more detail because of its low sample volume requirement and fast and inexpensive nature.

However, in order to broaden its use for studying populations, or even larger numbers of individuals, it may be useful to validate the method further. The major difficulty in developing the LESA-MS for milk triglycerides was the validation. The experimental barrier exists because it is difficult to impregnate an extant oil-in-water emulsion with internal standards. The introduction of organic solvents such as hexane or dichloromethane disrupts the hydrophobic effect that drives their formation in water, and production of synthetic oil bodies comprising lipid and triglyceride standards has yet to be realised.

Our review of the literature shows that this problem has been largely avoided in the validation of other methods. In most reports the internal standards were added after extraction of milk or in the extraction solvent[35], or no internal standard was used[6, 22, 32]. This is a valid approach because the data can be compared with the direct analysis of standards. We therefore limited validation to demonstrating repeatability of the method using quality control samples. This approach is limited in that it correlates closely with the concentration of the TG, with less abundant triglycerides proving less repeatable. Across the 30 samples we found that we could measure 23 triglycerides with a

coefficient of variance of <20%, regarded here as acceptable. The effect of this limit can mean that changes in concentration of triglycerides can be difficult to plot accurately. For example, the abundance of TG(54:3) may be around 0·01% two weeks *post partum*, but is about 3% at week 52. Without more thorough validation it is not possible to say whether it is produced from the outset or whether production begins in the first month *post partum*. However, such large relative changes in triglycerides have not been reported in so much detail yet, and so there is scope to build on these results and those of other formative studies[37].

As well as changes over time, there is a consistent difference between the milk produced in the two breasts at the same feed in the individual sample donors of the present study. Evidence from this study shows that one breast yields milk with a profile of higher shorter-chain (~C14) saturated fatty acids while the other breast shows a higher abundance of lipids with longer (~C18) unsaturated fatty acids. Also, the fragmentation data of the triglycerides shows differences in the composition of isobaric triglycerides.

This is perhaps surprising because the biosynthesis of FAs for milk fat production in the breast is generally in the range of 10-14 carbons per chain[38]. The breast that produces milk with a higher concentration of saturated shorter-chain fatty acids might be using higher amounts of sugars for *de novo* synthesis of FAs, whilst the other breast is more like a conduit for FAs from the circulation.

Another indication of the systemic import of FAs from the circulation is the qualitative details obtained by CID of the presence of very long saturated and mono-unsaturated fatty acids with C26:0 and C26:1 configurations. This is the first report on the presence of such very long chain fatty acids in human breast milk, as previous work reported up the presence of FAs up to C24. There was evidence for C26 FAs at all 3 time points, though appears to be clearer in the earlier samples (2 and 13 weeks). It is not clear why this should be, however as the abundance of a number of FAs changes through lactation, further work is required.

Further work is also required to understand the timing of the supply and the role of very long chain fatty acids supplied in breast milk. A number of studies show that C20 and C22 FAs have a role in neural development[35], cognition[36, 37] and behaviour[42]. The role(s) of C24 and C24:1 and even C26 in humans has received very limited attention and it is not clear if C24 and C26 are essential fatty acids[43]. These very long chain fatty acids are used in sphingolipid biosynthesis, which are essential in epidermal keratinocytes and male germ cells, along with much longer fatty acids (up to C36)[40, 41]. Humans are unable to make docosahexaenoic acid (DHA, C22:6$_{\omega\text{-}3}$) because we do not possess the appropriate dehydratases to biosynthesise C22:6 from C24:6[46]. What is known is that longer FARs, especially ones that are saturated or only singly unsaturated, form more ordered lipid aggregations[43, 44], which is consistent with the lateral inhomogeneity of liquid ordered regions and lipid rafts. The presence of such lipids therefore limits membrane fluidity.

It is important to emphasise that this is a proof-of-principle investigation based only on five individuals. These cannot be regarded as representative of Gambian or other women in general and these findings cannot be extrapolated. It is therefore not clear if these very long fatty acid in triglycerides are a common phenomenon in breast milk or if this is particular for these 5 individuals. However, as other studies that have collected milk samples from either mammary gland have shown that milk production varies between them[26, 27], the distinction between the triglyceride profiles of milk from different breasts during the same lactation may be more typical.

Nevertheless, more studies are required, comprising larger numbers of subjects from different populations, at different stages of lactation and different stages of a feed to further our understanding of milk production in humans.

Acknowledgements

The authors would like to thank Dr Ann Prentice for helpful discussions, Dr Landing M. A. Jarjou of the MRC International Nutrition Group in Keneba for supplying samples and to the MRC (SPT60) and the BBSRC (BB/M027252/1) for funding.

Author contributions

AK designed the research, carried out all experiments, analysed the data and wrote the proposal BB/M027252/1. AK and SF wrote the manuscript. MB advised on instrumentation and made instruments available. GG and LB provided conceptual input and supervision, and wrote the grant proposal (SPT60). AK wrote the grant proposal (BB/M027252/1). This was manuscript was already at an advanced stage when Dr Les Bluck sadly passed away, but his input and thoughts have been crucial to get this work together and we therefore want to include him as one of the authors.

References

[1] M. S. Kramer, R. Kakuma, *Cochrane Database Syst Rev* **2002**, CD003517.

[2] G. R. Goldberg, A. Prentice, A. Prentice, S. Filteau, K. Simondon, *Breast-Feeding: Early Influences on Later Health (Advances in Experimental Medicine and Biology)*, Springer, **2008**.

[3] SMCN, (Ed.: S. A. C. o. Nutrition), **2010**.

[4] W. H. O. WHO, in *55th World Health Assembly. Infant and Young Child Nutrition* (Ed.: WHA55.25), WHO, **2002**.

[5] F. E. Hytten, *British medical journal* **1954**, *1*, 175.

[6] G. Harzer, M. Haug, I. Dieterich, P. R. Gentner, *American Journal of Clinical Nutrition* **1983**, *37*, 612.

[7] R. G. Jensen, M. M. Hagerty, K. E. McMahon, *American Journal of Clinical Nutrition* **1978**, *31*, 990.

[8] M. C. Rudolph, M. C. Neville, S. M. Anderson, *Journal of Mammary Gland Biology and Neoplasia* **2007**, *12*, 269.

[9] M. C. Neville, R. P. Keller, J. Seacat, *American Journal of Clinical Nutrition* **1984**, *40*, 635.

[10] K. D. Dangat, S. S. Mehendale, H. R. Yadav, A. S. Kilari, A. V. Kulkarni, V. S. Taralekar, S. R. Joshi, *Neonatology* **2010**, *97*, 190.

[11] W. C. Breckenridge, A. Kuksis, *J Lipid Res* **1967**, *8*, 473.

[12] W. C. Breckenridge, A. Kuksis, *J Lipid Res* **1968**, *9*, 388.

[13] I. Haddad, M. Mozzon, R. Strabbioli, N. G. Frega, *International Dairy Journal* **2011**, *21*, 119.

[14] N. Spooner, R. Lad, M. Barfield, *Analytical Chemistry* **2009**, *81*, 1557.

[15] A. Koulman, P. Prentice, M. C. Y. Wong, L. Matthews, N. J. Bond, M. Eiden, J. L. Griffin, D. B. Dunger, *Metabolomics* **2014**, *10*, 1018.

[16] P. Prentice, A. Koulman, L. Matthews, C. L. Acerini, K. K. Ong, D. B. Dunger, *The Journal of Pediatrics* **2015**, *166*, 276.

[17] L. A. Lerno, J. B. German, C. B. Lebrilla, *Analytical Chemistry*, *82*, 4236.

[18] A. M. McAnoy, C. C. Wu, R. C. Murphy, *Journal of the American Society for Mass Spectrometry* **2005**, *16*, 1498.

[19] L. M. A. Jarjou, A. Prentice, Y. Sawo, M. A. Laskey, J. Bennett, G. R. Goldberg, T. J. Cole, *The American Journal of Clinical Nutrition* **2006**, *83*, 657.

[20] G. J. Van Berkel, V. Kertesz, *Anal Chem* **2009**, *81*, 9146.

[21] C. Molto-Puigmarti, A. I. Castellote, X. Carbonell-Estrany, M. C. Lopez-Sabater, *Clin Nutr* **2011**, *30*, 116.

[22] R. A. Gibson, G. M. Kneebone, *American Journal of Clinical Nutrition* **1981**, *34*, 252.

[23] C. J. Lammi-Keefe, R. G. Jensen, *Journal of Pediatric Gastroenterology and Nutrition* **1984**, *3*, 172.

[24] A. K. H. MacGibbon, M. W. Taylor, in *Advanced Dairy Chemistry Volume 2 Lipids* (Eds.: P. F. Fox, P. L. H. McSweeney), Springer US, Boston, MA, **2006**, pp. 1.

[25] M. M. Or-Rashid, N. E. Odongo, T. C. Wright, B. W. McBride, *Journal of Agricultural and Food Chemistry* **2009**, *57*, 1366.

[26] J. L. Engstrom, P. P. Meier, B. Jegier, J. E. Motykowski, J. L. Zuleger, *Breastfeeding Medicine* **2007**, *2*, 83.

[27] S. E. Daly, R. A. Owens, P. E. Hartmann, *Experimental Physiology* **1993**, *78*, 209.

[28] A. Prentice, A. M. Prentice, R. G. Whitehead, *Br J Nutr* **1981**, *45*, 483.

[29] S. E. Daly, A. Di Rosso, R. A. Owens, P. E. Hartmann, *Experimental Physiology* **1993**, *78*, 741.

[30] E. L. Jack, *Journal of Agricultural and Food Chemistry* **1960**, *8*, 377.

[31] S. M. Pons, A. C. Bargallo, C. C. Folgoso, M. C. Lopez Sabater, *European Journal of Clinical Nutrition* **2000**, *54*, 878.

[32] K. B. Nyuar, Y. Min, K. Ghebremeskel, A. K. Khalil, M. I. Elbashir, M. A. Cawford, *Acta Paediatr* **2010**, *99*, 1824.

[33] A. M. Ferris, R. G. Jensen, *Journal of Pediatric Gastroenterology and Nutrition* **1984**, *3*, 108.

[34] S. Salamon, J. Csapó, *Acta Universitatis Sapientiae - Alimentaria* **2009**, *2*, 196.

[35] E. R. Boersma, P. J. Offringa, F. A. Muskiet, W. M. Chase, I. J. Simmons, *Am J Clin Nutr* **1991**, *53*, 1197.

[36] W. D. Lassek, S. J. C. Gaulin, *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA)* **2014**, *91*, 195.

[37] M. Weiser, C. Butt, M. Mohajeri, *Nutrients* **2016**, *8*, 99.

[38] A. Crippa, C. Agostoni, M. Mauri, M. Molteni, M. Nobile, *Journal of Attention Disorders* **2016**.

[39] G. Yu, K. Duchén, B. Björkstén, *Acta Paediatrica, International Journal of Paediatrics* **1998**, *87*, 729.

[40] L. Govaerts, J. Bakkeren, L. Monnens, *Journal of Inherited Metabolic Disease* **1985**, *8*, 5.

[41] C. W. van Roermund, W. F. Visser, L. Ijlst, H. R. Waterham, R. J. Wanders, *Biochim Biophys Acta* **2011**, *1811*, 148.

[42] Y.-A. Moon, J. D. Horton, *Journal of Biological Chemistry* **2003**, *278*, 7335.

[43] R. Koynova, M. Caffrey, *Chemistry and Physics of Lipids* **1994**, *69*, 1.

[44] R. Koynova, M. Caffrey, *Biochimica et Biophysica Acta* **1998**, *1376*, 91.

Figures

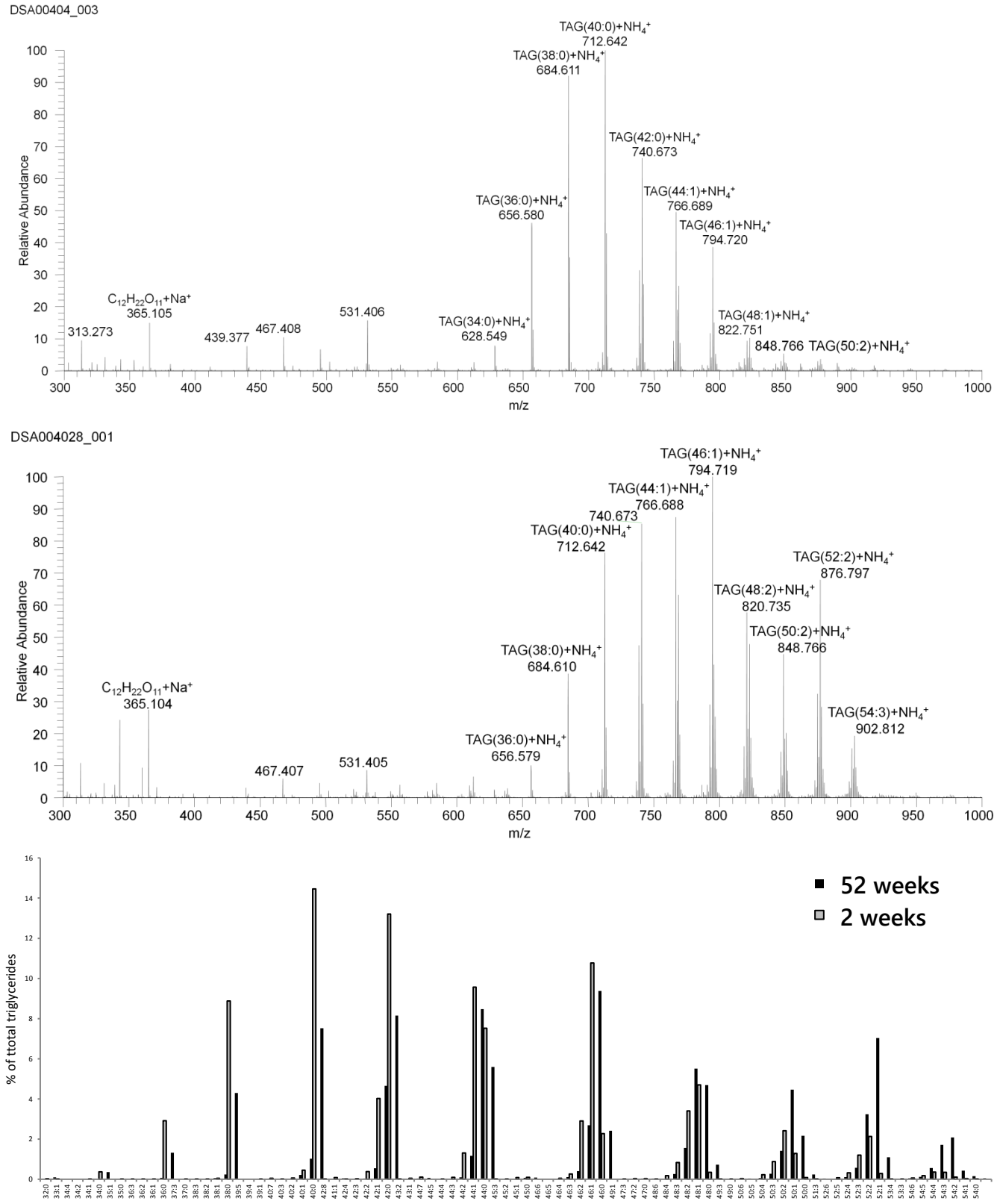

Fig. 1. LESA-MS[1] spectrum of an on-paper spotted sample of human breast milk collected at 2 weeks (top) and 52 weeks (middle) of lactation from one individual. Bottom bar graph shows the levels of each triglyceride signal as a percentage of total triglyceride signal.

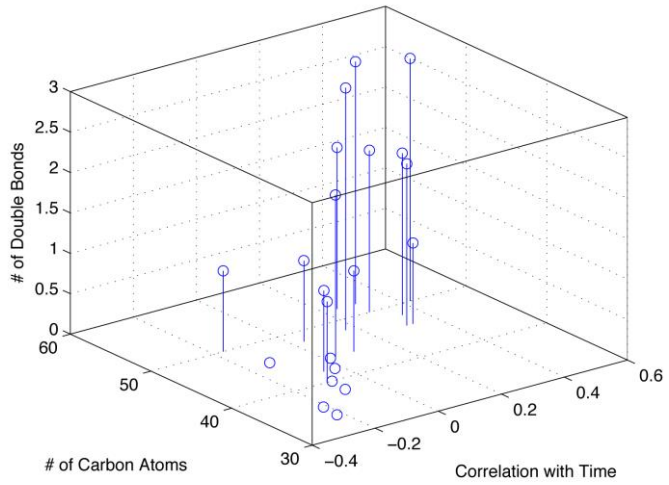

Fig 2. Correlation (x-axes) between stage of lactation (two and fifty weeks post partum*) and number of double (y-axes) bonds and number of carbons (z-axes).*

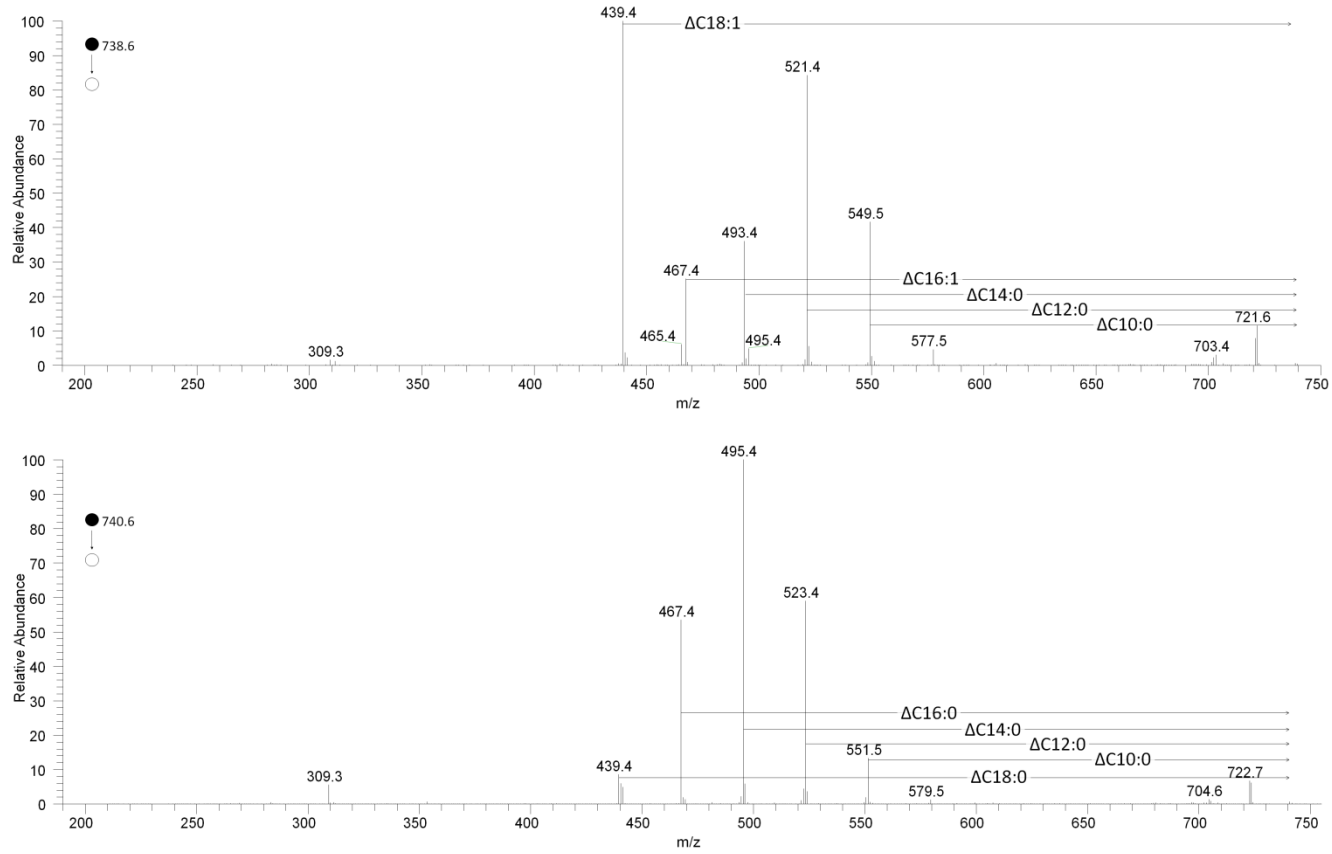

Fig. 3. MS^2 spectra of the ammoniated ions of TG(42:1) (top trace) and TG(42:0) bottom trace.

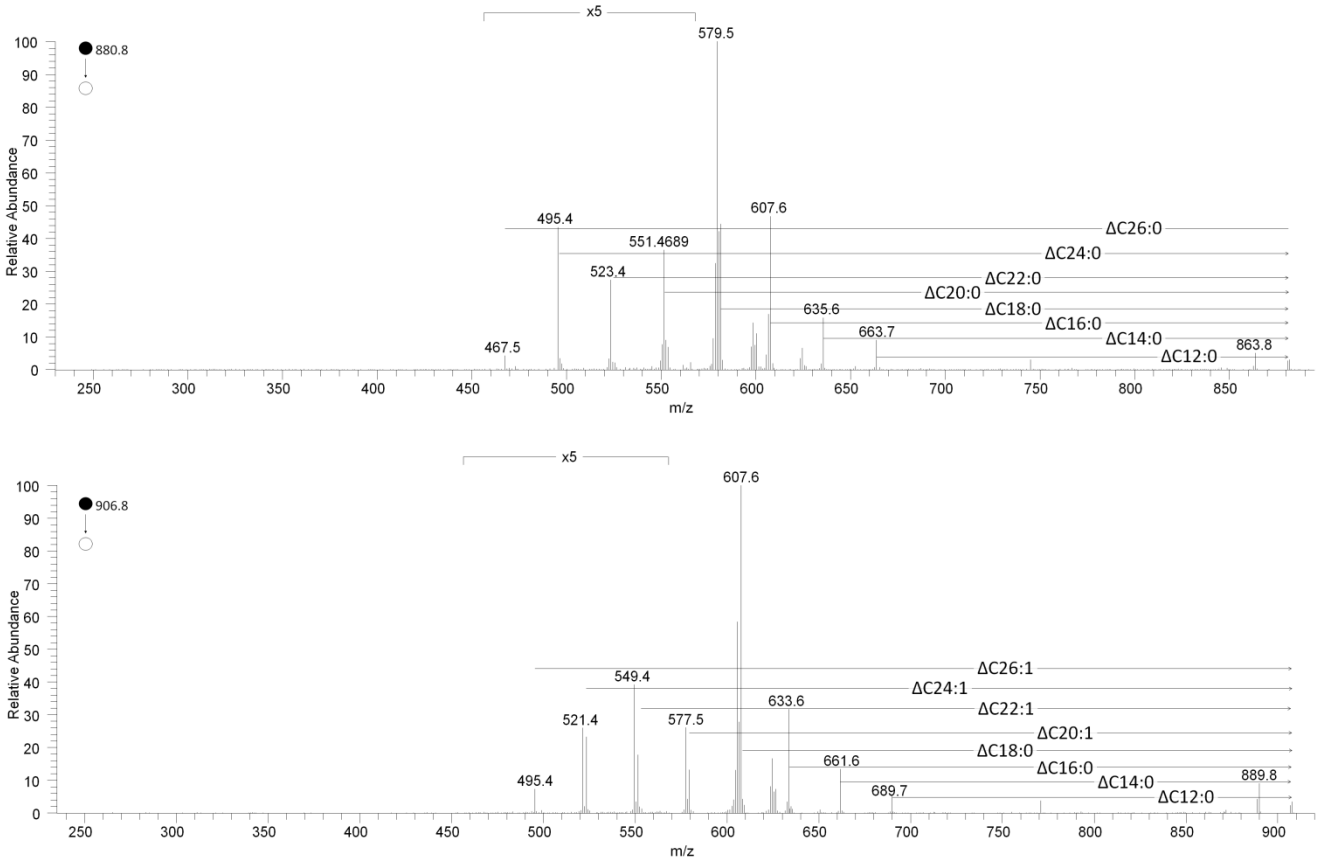

Fig. 4. The MS^2 spectra of TG(52:0) (top graph) and TG(54:1) showing the relative abundance of the different fatty acids contributing to these triglycerides. This has not previously been reported in human milk samples.

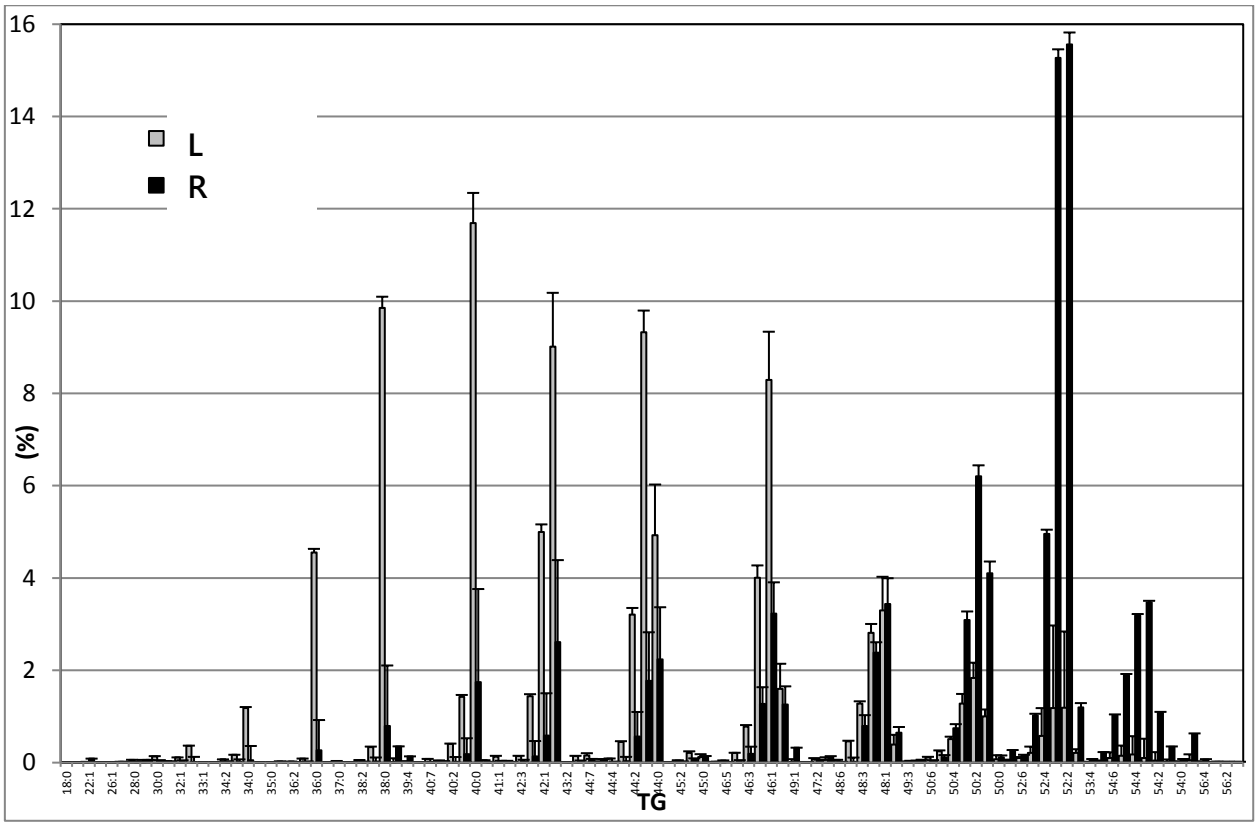

Fig 5. The average relative values of triglycerides in human breast milk collected from the two breasts at 13 weeks of lactation in one subject: left (grey bars) and right (black bars). n = *4.*