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Towards the identification of metabolite markers of nipple pain and inflammation in human milk

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2 Materials and Methods

2.1 Materials

All materials used in the methods, are presented in (the following) tables, 2.1-2.5. Materials are separated into four tables to reflect the four sections presented in the methods. The fifth table contains the identification of metabolite reference standards used for metabolomic optimisation. Materials are presented with supplier and abbreviations where possible and suppliers are arranged in the order they appear in Methods.

Reagents were prepared using double deionized water (DDI) (supplied by The Hartmann Human Lactation Research Group, UWA) unless stated otherwise. DDI water was prepared with a PRELAB Classic water purification system (Ibis Technology, Osborne Park, WA, Australia); the DDI water used for breast sterilisation was further heat sterilised using an autoclave.

Table 2.1 Human milk collection method, with supplier listed.

Chemical
Thermo Fisher Scientific Australia Pty. Ltd.
PDI Alcohol prep pads (70% isopropyl)
Medical and Surgical Requisites Pty Ltd, QLD, Australia
5% Chlorhexidine skin cleanser (Microshield)

Table 2.2 Bacteriology methods, with supplier and abbreviations listed.

Chemical		Abbreviation
Pathwest Laboratory Medicine WA, Forrest House Mt Claremont, WA, Australia		
5% Horse blood agar plates (PO81)		
Deoxyribonuclease (DNase) agar plates (P090)		
Sabouraud Dextrose agar powder (Oxoid- CM0041)		
Vogel-Johnson agar plates (1347)		
Yeast Extract Glucose Chloramphenicol (YGC) agar (1498)		
Dichloran Rose Bengal Chlortetracycline (DRBC) agar plates (1139)		
Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia		
Mannitol Salt Phenol Red agar powder (63567)		
Amber Scientific, Midvale, WA, Australia		
Gram stain kit (ref#-76):	Crystal violet 0.5% stain (CV-500)	GV
	Gram's iodide solution (GI-500)	GI
	Decoulourizer solution (DECL-500)	DECL
	Safarin aqueous stain (SF-500)	SF
Blackaby Diagnostics, Darlington, WA, Australia		
Phadebact latex kit		
Thermo Fisher Scientific Australia Pty Ltd, Melbourne, VIC, Australia		
Hydrochloric acid (1N) (SA48-1)		HCl

Table 2.3 Sodium and potassium analysis, with supplier and abbreviations listed.

Chemical	Abbreviation
Univar ®, Redmond, WA, USA	
Sodium chloride (465)	NaCl
Potassium chloride (383)	KCl

Table 2.4 Metabolomic analysis of human and bovine milk, with supplier and abbreviations listed.

Chemical	Abbreviation
Thermo Fisher Scientific Australia Pty. Ltd., Melbourne, VIC, Australia	
Methanol (LCMS grade) (A454-4)	MeOH
Water (LCMS grade) (W6-4)	
Acetonitrile (A955-4)	ACN
Hexane 95% (H306-4)	
Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia	
D-Sorbitol-$^{13}C_6$ 99% (605514)	
N-Methyl-N-(trimethylsilyl) trifluoroacetamide(M7891)(394866)	MSTFA
Methoxyamine hydrochloride 98% (226904)	
2-Aminoanthracene (A38800)	
Ajax Finechem, Sydney, NSW, Australia	
Pyridine (AJA430)	
Chem Service Inc., West Chester, PA, USA	
n-decane C_{10} (0-729) (Purity – 99.8%)	
n-dodecane C_{12} (0-731) (Purity - 99.4%)	
n-pentadecane C_{15} (0-2238) (Purity - 98.9%)	
n-nonadecane C_{19} (0-2203) (Purity – 99.4%)	
n-docosane C_{22} (0-2089) (Purity – 99.2%)	
n-octacosane C_{28} (0-2227) (Purity- 99.5%)	
n-dotriacontane C_{32} (0-2095) (Purity - 98.5%)	
n-hexatriacontane C_{36} (0-2128) (Purity - 98%)	
Grace Davison Discovery Sciences, Rowville, VIC, Australia	
SPE bulk sorbent prevail C_{18} (%125474)	SPE

Table 2.5 List of metabolite reference standards used for metabolomic method optimisation, with supplier listed.

Metabolite
Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
L-Valine (V0500) (Purity 98%)
L- Alanine (A7627) (Purity 98%)
Glycine (241261) (Purity 99%)
L- Leucine (L8000) (Purity 98%)
L-Proline (P0380) (Purity 99%)
L-Isoleucine (I2752) (Purity 98%)
L-Norleucine (N6877) (Purity 98%)
L-Cysteine (168149) (Purity 98%)
Urea (U5378)
L-Serine (S4500) (Purity 99%)
Ethanolamine (E9508) (Purity 98%)
L-Threonine (T8625) (Purity 98%)
Succinic Acid (398055) (Purity 99%)
Putrescine (dihyrdochloride) (P7505) (Purity 98%)
L-Serine (S4500) (Purity 99%)
L-Methionine (M9625) (Purity 98%)
L-Aspartic acid (A9256) (Purity 98%)
L-Glutamic Acid (G1251) (Purity 99%)
L-Phenylalanine (P2126) (Purity 98%)
a-Ketoglutaric acid (K1750) (Purity 98.5%)
Ribitol (Adonitol) (A5502) (Purity 99%)
Citric acid (251275) (Purity 99.5%)
L-Lysine (L5501) (Purity 98%)
D-(-)-Fructose (F0127) (Purity 99%)
L-Tyrosine (W373605) (Purity 97%)
D-Mannitol (M4125) (Purity 98%)
D-Sorbitol (S1876) (Purity 98%)
Myo-inositol (I5125) (Purity 99%)
Ribose-5-phosphate (disodium salt hydrate) (83875) (Purity 99%)
L-Tryptophan (T0254) (Purity 98%)

Table 2.6 Equipment used, with manufacturer listed.

Equipment
Medela AG, Baar, Switzerland
Symphony® breast pump and attachments
Quick Clean™ microwave bags
Corning Incorporated, Corning, NY, USA
PC-351 HOT Plate Stirrer
Getinge AB Group, Getinge, Sweden
HS4406 Steam Sterilizer (autoclave)
Forma Scientific, Inc., Marietta, OH, USA
3164 - Water Jacketed Incubator
Horiba Scientific Ltd., Kyoto, Japan
LAQUAtwin sodium ion electrode (S022)
LAQUAtwin potassium ion electrode (S030)
Crown Scientific Pty Ltd., NSW, Australia
Eppendorf Thermomixer® comfort
Microcentrifuge 5415R
IKA MS1 Works (vortex)
Bruker Daltonics, Billerica, MA, USA
450-GC Oven
Shimadzu Corporation, Kyoto, Japan
GC-MS- Shimadzu QP2010 Ultra, Kyoto, Japan

Table 2.7 Data analysis software, with manufacturer listed.

Software
R Development Core Team R 3.0.3 GUI 1.638 Snow Leopard build 32-bit (6660)
SpectralWorks Ltd, Cheshire, United Kingdom AnalyzerPro 2.7.0.0
Shimadzu Corporation, Kyoto, Japan GCMSsolution 2.61
Camo Software AS The Unscrambler
SAS Institute Inc. JMP 8.0.2

2.2 Methods

2.2.1 Human sample collection

2.2.1.1 Recruitment

Mothers between the ages of 18-45 years currently breastfeeding a baby between the age of 1-6 months were recruited. Participants were predominantly recruited through community engagement and ongoing communication using flyers, World Wide Web (e.g. social media) and in person promotion. Additional participants were recruited via the Australian Breastfeeding Association (ABA), local community health nurses and lactation consultants and through posts on the University of Western Australia website. Mothers already participating in research studies associated with Hartmann Human Lactation Research Group (HHLRG) under the supervision of Professor Peter Hartmann were also invited to participate.

Mothers were supplied with a consent form and general information sheet to complete at the time of milk collection (Appendix 2.1 and 2.2). Using the Visual Analogue Scale (VAS) for pain intensity as described by McClellan et al. (2012) mothers were asked to rate their pain when breastfeeding from 'no pain' to the 'worst pain imaginable'. Measured pain intensity was recorded and converted to a numerical value between 0 and 10, 0 being no pain and 10 representing the highest level of perceived pain.

Mothers were registered online with the HHLRG and provided with a unique identification number. Mothers received their identification number via email which contained a link directing them to an online questionnaire with questions relating to

parity, infant and maternal age, medication and current health status of the mother and infant. The questionnaire is provided in Appendix 2.3.

2.2.1.2 Human milk collection

Milk samples were collected from mothers with and without persistent nipple pain at a single time-point. Milk samples were collected from a single breast in mothers without nipple pain (control). Mothers with bilateral nipple pain provided a single milk sample from the breast experiencing the greatest level of perceived pain during breastfeeding. Mothers with unilateral nipple pain were asked to provide a sample from the affected breast and non-affected breast; this acted as a paired control.

Participating mothers were requested not to express or breastfeed for at least 3 hours prior to milk collection (most relevant to those presenting with unilateral nipple pain and therefore suitable to donate both a nipple pain and control sample) to ensure the breast was full or near full at the time of collection (Hassiotou et al, 2013), and/or required to provide a sample from the breast that had not recently expressed. Before collection mothers were required to adequately clean their hands (using Aqium antibacterial hand gel) and their breast to remove skin contaminants. The breast was washed by rinsing the nipple and surrounding skin with sterile water (DDI water that had been sterilised by autoclave at 120 °C for 15 minutes), followed by an alcohol wipe (70 % isopropyl alcohol) (Thermo Fisher Scientific Australia Pty Ltd.), a mild disinfectant (chlorhexidine) (Medical and Surgical Requisites Pty Ltd) and then a final rinse with sterile water before milk collection (Hale, Bateman, Finkelman & Berens, 2009). The breast pump and its attachments were sterilised in Quick Clean ™ microwave sterilising bags (Medela, AG) prior to use. Participating mothers were

fitted with a breast shield connected to a Symphony® electric breast pump (Medela AG) and instructed to set the pump to a setting comfortable for them. A minimum of 15 ml of breast milk was collected and immediately put on ice, covered from light and transported to the laboratory for storage.

On arrival at the laboratory the samples were gently mixed and divided into three aliquots of 5 ml into 15 ml falcon tubes and stored at -80 °C until analysis.

For sample analysis purposes human participants were de-identified and given reference identification numbers, mothers were labelled M01-M29 with the addition of sample identification numbers separating the control (C01-C22) and NP (NP01-NP11) participants.

2.2.1.3 Participant demographic data analysis

Analyses of participant demographics were conducted to identify differences between control and nipple pain subgroups. Participant identification numbers and sample identification numbers are presented in the tables in the appendix.

Demographic analysis focused on maternal age, infant age and parity. All analyses were performed using R 3.0.3 GUI 1.638 Snow Leopard for Mac OSX (R Development Core Team, 2011) using the base packages, and the libraries NLME (Pinheiro, Bates, DebRoy & Sarkar, 2011) and multcomp (Hothorn, Bretz & Westfall, 2008), which were used for linear mixed modelling and multiple comparison of means, respectively. A linear mixed effect model and linear regression (linear model) were calculated and compared by ANOVA to determine underlying physiological

differences between individuals. Categorical variables, including parity, were compared using Fisher's exact test. Summary statistics are presented as mean ± SD, or median (IQR), or proportion. $p < 0.05$ was considered to be statistically significant.

2.2.2 Bovine sample collection

A set of bovine milk samples, from healthy cows (control), cows suffering from mastitis and pooled samples from a single storage vat, were collected for comparative purpose to be used as positive control throughout the study. Milk samples were collected from Friesian Holstein (Black and White) dairy cows at a functional dairy in the south west of Western Australia. Pooled milk samples were collected by removing a 15 mL aliquot from a single storage vat prior to pasteurisation. Samples from individual cows were collected by hand expression of 15 ml of milk into a 25 ml falcon tube. All samples were sealed, covered from light, stored on ice and immediately returned to Perth for storage in an -80 °C freezer.

Bovine milk samples were collected from four lactating cows, given reference identification numbers B01-04, and the pooled bovine vat collections were labelled V01-V02. Additionally individual bovine samples were identified by sample identification numbers BC01-03 for bovine control samples, BM01-04 for samples retrieved from cows with mastitis and BV01-BV02 for the two pooled bovine vat samples.

2.2.3 Microbial methods

2.2.3.1 Media preparation

Mannitol Salt agar

Mannitol salt phenol red agar powder (Sigma-Aldrich Pty. Ltd., Australia) 111 g/L was prepared (in Millipore filtered double deionised distilled water). Agar solution was sterilize by autoclave (HS4406 Steam Sterilizer) (Getinge AB Group, Sweden) at 121 °C for 15 minutes and stored in 100 mm plates (50 plates, 25 ml per plate) below 8 °C.

Sabouraud Dextrose agar

Sabouraud Dextrose agar powder (Sigma-Aldrich Pty Ltd., Australia) 65 g/L was prepared (in Millipore filtered double deionised distilled water) and brought to boiling point until dissolved, using a PC-351 HOT plate Stirrer (Corning Incomprated, USA). Agar solution was sterilize by autoclaving (HS4406 Steam Sterilizer) (Getinge AB Group, Sweden) at 121 °C for 15 minutes and poured into 100 mm plates (50 plates, 25 ml per plate) below 8 °C.

2.2.3.2 Culture

Bacterial cultures were prepared in a laminar flow hood under sterile condition as recommended by the National Mastitis council (2012). 50 µl of each milk sample were spread evenly on each of the following agar plates in dublicate; 5 % Horse blood agar, Mannitol Salt agar (MSA) and Sabouraud Dextrose agar. Plates were incubated (3164 - Water Jacketed Incubator, Forma Scientific, USA) at 37 °C for 24 ± 2 hours; Sabouraud agar plates were incubated for an additional 96 hours.

A colony forming unit (CFU) count was conducted to determine total CFU/ml on 5% Horse Blood agar, MSA and Sabouraud agar. Bacterial species were identified by Gram stain with the help of Cristina Farrar (Senior Medical Scientist at Princess Margaret Hospital, Subiaco W.A.). *S. aureus* was identified based on positive latex agglutination test (to detect the coagulase enzyme) and positive culture on DNase *S. aureus* specific agar plate.

Additional agar plates Dichloran Rose Bengal Chlortetracycline (DRBC) agar and Yeast Extract Glucose Chloramphenicol (YGC) agar (supplied by PMH) were used for specific isolation and identification of yeast species and Vogel-Johnson agar was used for the isolation and quantification of *S. aureus* following previous identification of target species (yeast and *S. aureus)* on Sabouraud agar and MSA respectively. All cultures were prepared following previously mentioned culture techniques, i.e. 50 µl of whole milk spread evenly on the agar plate and incubated at 37 °C for 24 ± 2 hours.

2.2.3.3 Bacterial and fungal species detection through Gram stain

Traditional Gram stain technique, as adapted from the original publication of Gram (1984) was used for microbial identification (Gram, 1884; Gephart et al., 1981). The colony of interest was transferred by loop onto a clean glass slide and heat fixed prior to applying a Gram stain. Using a Gram stain kit (Amber Scientific, Australia) the slide was flooded with crystal violet solution for up to a minute, washed briefly with distilled water and the excess water drained. Using Gram's iodide solution the slide was flooded again and left to sit for one minute before washing with distilled water

and draining. Using paper towel the slide was gently blotted dry and flooded for 10 seconds with decolourizer solution followed by washing with distilled water and draining. Lastly the slide was flooded with Safranin aqueous stain for 30 seconds. The slide was then washed using distilled water, followed by draining and blotting dry (being careful not to rub).

All slides were examined under an oil immersion lens and bacterial species were identified.

2.2.3.4 S. aureus identification

2.2.3.4.1 Latex agglutination test

The latex agglutination test was conducted to confirm the presence of *S. aureus* using a latex agglutination kit (Blackaby Diagnostics, Australia); following culture on blood agar and MSA.

Once a potential culture of *S. aureus* was identified on an MSA plate an inoculation loop was used to transfer a single colony to a latex agglutination card. A drop of red latex was added and the solution was mixed thoroughly using an inoculation loop followed by gently hand rocking the card for a further 20 seconds. Agglutination or clumping should be instantaneous with most *S. aureus* strains.

2.2.3.4.2 DNase agar plate

Additionally any potential *S. aureus* cultures identified on MSA plates were cultured on a DNase agar plate (supplied by Pathwest Laboratory Medicine, Australia). Using

an inoculation loop a single colony was transferred to a DNase agar plate and spread in a small circle on the surface of the agar. The plate was incubated (3164 - Water Jacketed Incubator, Forma Scientific, USA) for 24 hours at 37 °C followed by the addition of 1N HCl (Thermo Fisher Scientific Australia Pty Ltd.). The addition of HCl precipitates DNA in the media causing cloudiness, unless the DNA has been hydrolysed by *S. aureus* in which case creating a clear zone or 'halo' around the growing colony occurs, which indicates positive growth for *S. aureus*.

2.2.3.5 Data analysis

Data analyses of CFU/ml (as determined by culture on 5% horse blood agar) was performed using R 3.0.3 GUI 1.638 Snow Leopard for Mac OSX (R Development Core Team, 2011) using the base packages, and the libraries NLME (Pinheiro et al., 2011) and multcomp (Hothorn et al., 2008), which were used for linear mixed modelling and multiple comparison of means, respectively. Summary statistics are presented as mean ± SD of the CFU/ml and logarithmic value of the CFU/ml. A p-value < 0.05 was considered to be statistically significant.

2.2.4 Sodium and potassium analysis

Sodium ($Na^{+)}$ and potassium (K^{+}) ion analysis was based on a previous method using ion electrodes by Fetherston, Lai and Hartmann (2006). To determine the accuracy and reproducibility of the ion electrodes a set of concentration standards and calibration recovery standards were prepared and analysed prior to the analyses of the final sample set. Quality controls were used to identify unwanted variation such as the result of preparation or instrumental error (De Livera et al., 2012). Additional concentration standards and calibration recovery standards were analysed throughout

the final sample set to ensure that reproducibility within ± 10 % was being maintained.

Na^+ and K^+ concentration was measured using Horiba LAQUAtwin sodium ion electrode (S022) and Horiba LAQUAtwin potassium ion electrode (S030) (Horiba Scientific Ltd.). 300 µl from each sample was transferred by pipette onto the electrode and the reading was recorded (mV). The sample was removed and discarded and the electrode was washed using DDI water and dried with blotting paper and the process repeated. This method was used for the final sample set.

2.2.4.1 Preparation of concentration standards

A total of 10 sets of 7 preparation standards of known concentration were prepared, one set was used for method optimisation and the remainder were used for analysis of the final sample set.

A 25mM sodium/potassium (Na/K) stock was prepared by dissolving 1.461 g/L of NaCl (Univar ®, USA) and 1.964 g/L of KCl (Univar ®, USA) in 100 ml of (DDI) water. Using the 25 mM stock, Na/K standards between 0.5-25 mM were prepared in 15 ml falcon tubes as per Table 2.8.

The standards were divided into 1.5 ml aliquots using 2 ml centrifuge tubes and stored at -20 °C before use. Aliquots of the standards were used later to create a sodium and potassium standard curve to assist with calculation of sodium and potassium ion concentration in actual milk samples.

Table 2.8 Preparation of sodium and potassium standards for a standard curve.

Concentration (mM)	25mM Stock (ml)	DDI (ml)
0.5	0.2	9.8
2.5	1.0	9.0
5.0	2.0	8.0
10.0	4.0	6.0
12.5	5.0	5.0
17.5	7.0	3.0
25.0	10	0

2.2.4.2 Preparation of calibration recovery standards

A total of 10 sets of 3 calibration recovery standards was prepared, 3 sets were used for method optimisation and the remainder were used as part of the analysis of the final sample set.

A 15 mM Na/K stock standard was prepared in a 25 ml falcon tube by adding 12 ml of the previously made 25 mM Na/K stock to 8 ml of DDI. Using the 15 mM Na/K stock 10 sets (3 test tubes per set) of calibration recovery standards were dispensed in 2 ml centrifuge tubes as seen in Table 2.9. All aliquots were stored at -20°C.

Table 2.9 Preparation of calibration recovery standards.

Test tube	
1	750 µl of whole milk + 750 µl of 15 mM standard
2	750 µl of whole milk + 750 µl of DDI
3	750 µl of DDI + 750 µl of 15 mM standard

2.2.4.3 Method optimisation: recovery assay for calibration recovery standards

One full set of Na^+/K^+ standards (seven tubes) and three sets of calibration recovery standards (nine tubes) were removed from the freezer and incubated at room temperature for 5 minutes. Na^+ and K^+ concentrations in mV was measured and recorded in duplicate using the previously described method (2.2.4).

A logarithmic standard curve was prepared for the Na^+ and K^+ concentration standards. This was plotted on an XY graph (Figure 2.1 and Figure 2.2) and used to determine the unknown concentrations of the calibration recovery standards.

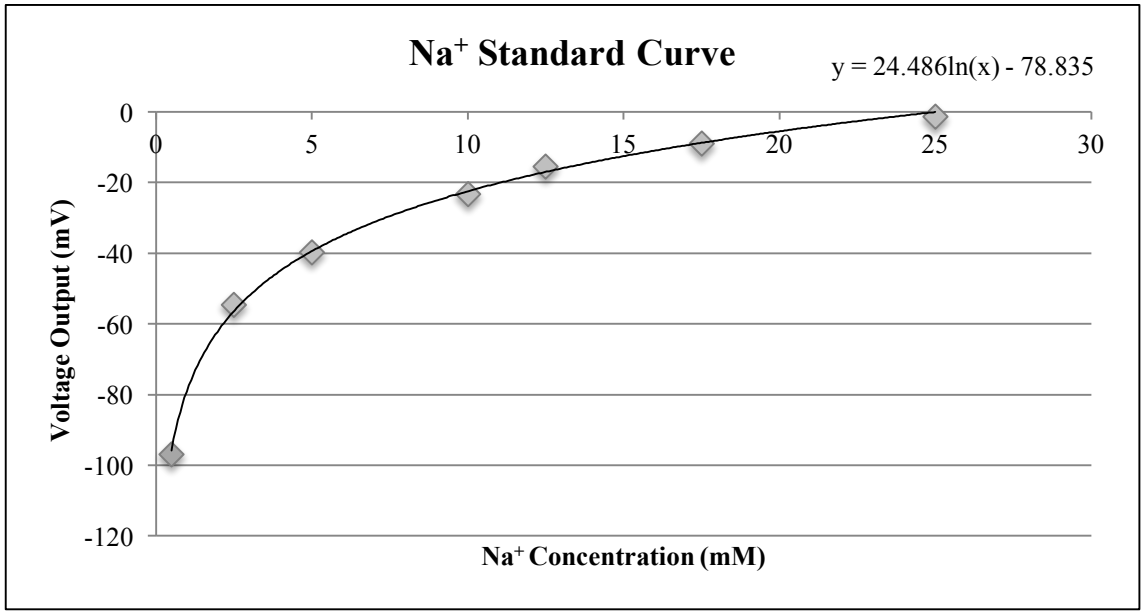

Figure 2.1 Na^+ standard curve and logarithmic equation.

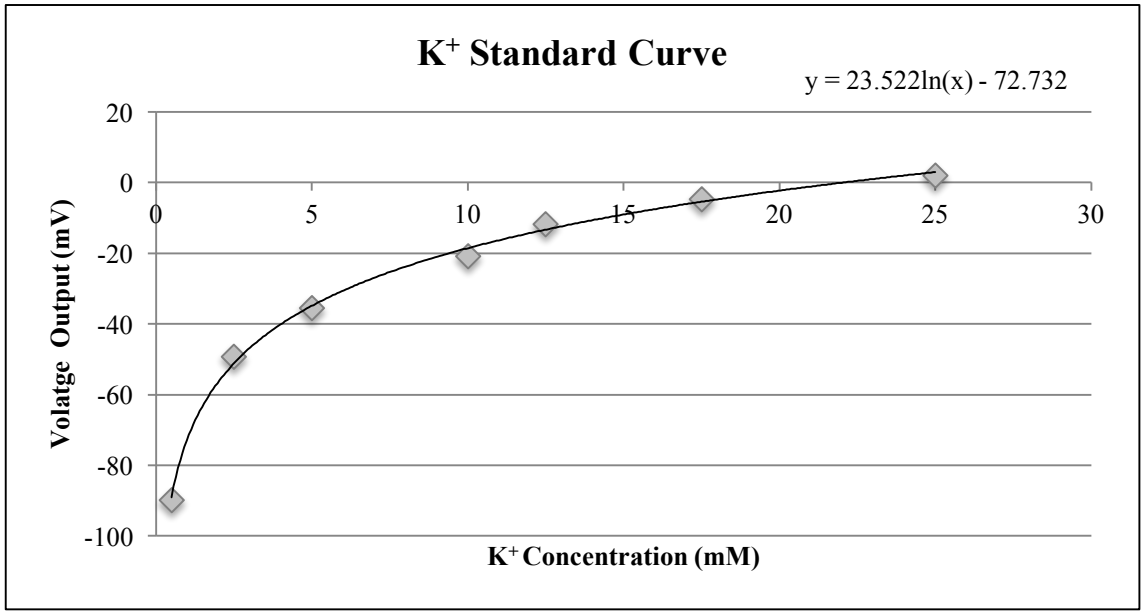

Figure 2.2 K^+ standard curve and logarithmic equation.

To determine the accuracy and reproducibility of the electrodes, the calculation as seen in Figure 2.3 was used for each set of calibration recovery standards using the average concentration of the individual aliquot duplicates. A reproducibility of ± 10% was considered acceptable.

$$\frac{[\textbf{ONE}\ (\text{mM}) - \textbf{TWO}\ (\text{mM})]}{\textbf{THREE}\ (\text{mM})} = 1.00 = 100\%$$

Example Calculation:

Tube one: 13.59 mM
Tube two: 7.05 mM
Tube three: 6.43

$$\frac{13.59 - 7.05}{6.43} = 1.02 = 102\%$$

Figure 2.3 Reproducibility calculations for calibration recovery standards.
Values ONE, TWO and THREE correspond with calibration recovery standards test tubes 1, 2 and 3 in Table 3.2.

Table 2.10 illustrates the reproducibility calculated for the initial three sets of calibration recovery standards. All sets produced reproducibility within ± 10 % and only five measurements displayed reproducibility less than ± 5 % (represented by the shaded cells in Table 2.10). These results were deemed satisfactory to move onto subsequent analysis.

Table 2.10 Reproducibility of calibration recovery standards.

Recovery set	Ion	Aliquot A	Aliquot B	Aliquot C
1	Na^+	1.02	0.99	1.01
	K^+	1.02	1.00	1.00
2	Na^+	0.99	0.97	1.04
	K^+	0.94	0.96	1.10
3	Na^+	0.95	1.00	0.92
	K^+	0.94	1.07	1.02

Reproducibility of less than ± 10 % is required for each set of calibration recovery standards.
Most samples had a % recovery less than 5 %.
Highlighted cells indicate reproducibility ≥ ± 5 %.

2.2.4.4 Measurement of Na^+ and K^+ ion concentration in milk

A set of concentration standards and calibration recovery standards were analysed each day prior to milk sample analysis. The calibration recovery standards were used to ensure reproducibility was being maintained and the concentration standards were used to calculate a logarithmic equation used to determine the Na^+/K^+ concentrations of the samples analysed on that day (total of four days of analysis).

The Na^+ and K^+ concentration of each milk sample was measured in mV and recorded in duplicate using the previously described method (2.2.4.). Readings were converted from mV to mM using the equation calculated from the sodium and potassium standards as previously explained. Once all Na^+ and K^+ measurements were converted to mM the Na^+/K^+ ratio was calculated [by dividing the Na^+ concentration by the K^+ concentration]. A Na^+/K^+ ratio level above 1 was considered abnormal and a sign of inflammation or possible infection (Aryeetey et al., 2008).

Following analysis of all milk samples and corresponding standards the equations for all standard curves was compared to identify any differences in concentrations that may have occurred between days (Figure 2.4 and Figure 2.5).

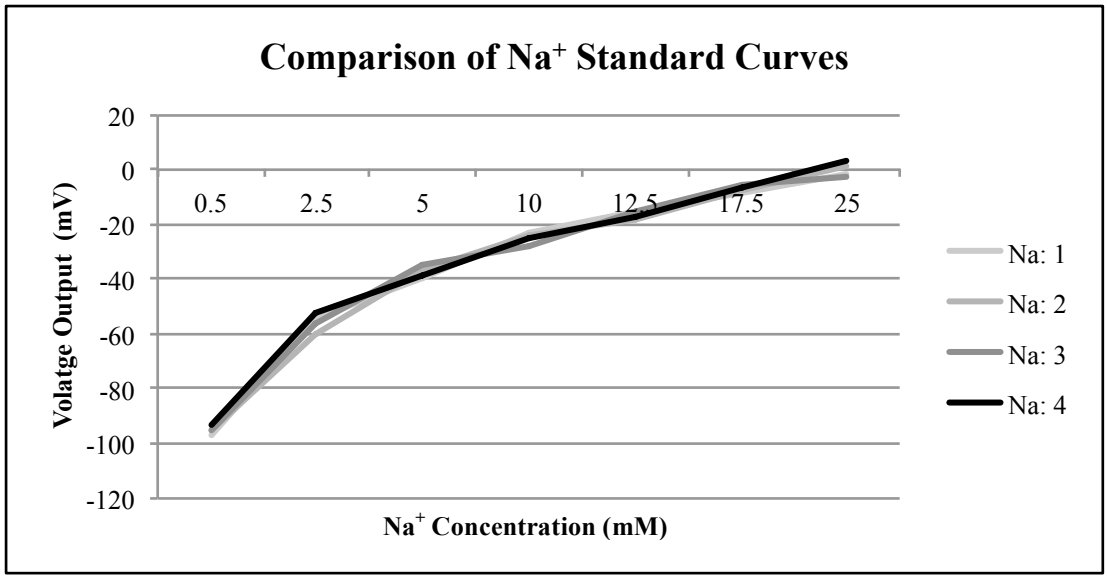

Figure 2.4 Comparison of Na^+ standard curves.
This figure shows the reproducibility of the Na^+ standard curve as measured on four separate occasions.

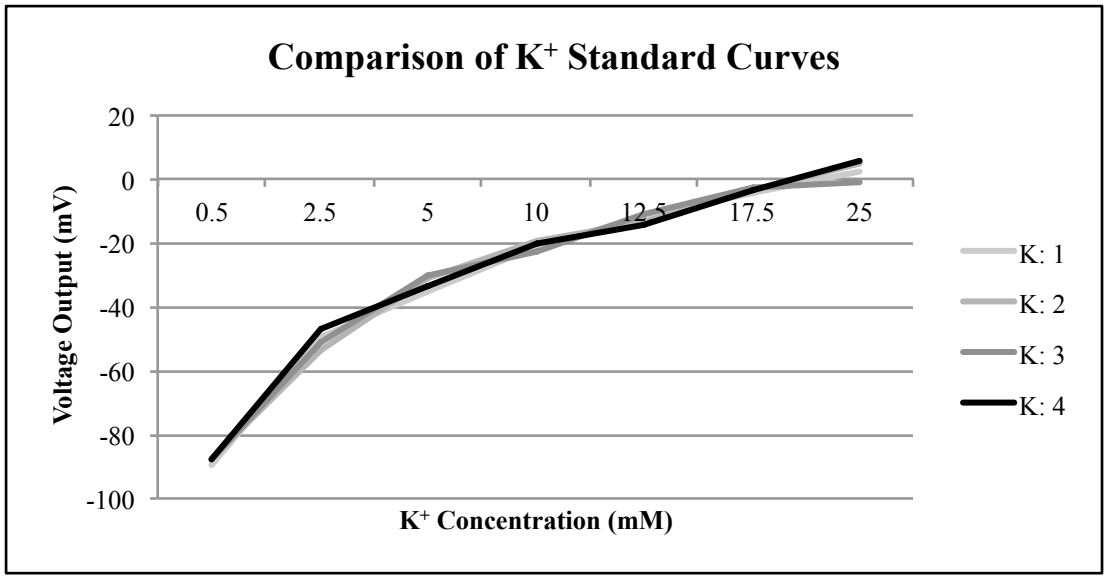

Figure 2.5 Comparison of K^+ standard curves.
This figure shows the reproducibility of the K^+ standard curve as measured on four separate occasions.

2.2.4.5 Data analysis

All samples were analysed for Na^+ and K^+ concentration and Na^+/K^+ ratios were calculated. All analyses were performed using R 3.0.3 GUI 1.638 Snow Leopard for Mac OSX (R Development Core Team, 2011) using the base packages, and the libraries NLME (Pinheiro et al., 2011) and multcomp (Hothorn et al., 2008), which were used for linear mixed modelling and multiple comparison of means, respectively. A linear mixed effect model and linear regression (linear model) were calculated and compared by ANOVA to determine underlying physiological differences between individuals. Summary statistics are presented as mean ± SD. A p-value < 0.05 was considered to be statistically significant.

2.2.5 Metabolomic profiling of human and bovine milk

Due to the complexity of human milk, together with the large variability between individuals, and the inherent difference between milk and other biological matrices, key components of the methods were optimised for the measurement of milk metabolites. The components requiring optimisation were the sample volume and the derivatisation reaction temperature.

Derivatisation allows the analysis of compounds that are not directly amenable to GC analysis. An increased volatility is one such benefit of derivatisation, and particularly for early eluting compounds, improved volatility can permit analysis in an otherwise obscured chromatographic background (Gullberg, Jonsson, Nordström, Sjöström, & Moritz, 2004). Within the literature a range of derivatisation reactions and conditions including temperatures and derivatisation reaction times has been used previously (Gummer, Trengove, Oliver and Solomon, 2013; Gullberg et al., 2004; Dunn et al., 2011; Bressanello et al., 2014).

Therefore, sample preparation optimisation, namely the incubation temperature for MSTFA derivatisation, and the optimal sample volume that can be analysed in a single derivatising volume, was carried out prior to the analyses of the final sample set.

2.2.5.1 Metabolite analyses

Polar metabolites were isolated from the milk using a solid phase extraction approach, following removal of the protein content by organic solvent precipitation. The dried

extract was then derivatised using a combination of oximation and silylation in preparation for instrumental analysis.

Proteins were precipitated by the addition of 1.2 mL ACN, added whilst gently mixing, and incubated at room temperature for 10 minutes. The precipitate was pelleted by centrifuging for 20 minutes at 10°C at 16,100 g. The supernatant (1,600 µl) was transferred into a fresh 2 ml centrifuge tube and the remaining pellet discarded. The supernatants were divided into two aliquots of 180 µl (equivalent to 50 µl of milk), which were frozen on dry ice and dried by lyophilisation. The remaining volume was stored at -80°C.

Samples were redissolved and fractioned by SPE according to the methods of Gummer and Trengove (Unpublished 2015). Specific details are not available due to intellectual property issues regarding patent of this method. Briefly, the method involves separating polar metabolites from non-polar. The polar fraction was then dried by lyophilisation in preparation for derivatisation. The remaining non-polar components were stored at -80°C for future studies.

Dried samples were derivatised in batches of 24, including QC samples, based on the methods of Gummer et al. (2013), but further optimised for milk analysis (in this thesis). A methoxyamine solution was prepared by dissolving 20 mg/ml of methoxyamine hydrochloride (Sigma-Aldrich Pty. Ltd.) in pyridine (Ajax Finechem). 20 µl was added to each sample and agitated at 1,200 rpm for 90 minutes at 30°C using an Eppendorf Thermomixer® Comfort (Crown Scientific Pty Ltd.). Samples were then centrifuged in a Microcentrifuge 5415R (Crown Scientific Pty Ltd.) for one minute at 18 °C and 16,100 g. The methoxymated sample solution was transferred to a glass analytical vial with a glass insert followed by the addition of a 5 µl solution of

n-alkanes $C_{10,}$ C_{12}, C_{15}, C_{19}, C_{22}, C_{28}, C_{32}, C_{36} (Chem Service Inc., USA), in n-hexane (95 %) (Thermo Fisher Scientific Australia Pty Ltd) for calculation of retention indices, and 40 µl of MSTFA (Sigma-Aldrich Pty. Ltd.). The vials were cap sealed and gently mixed using a vortex, IKA MS1 Works (Crown Scientific Pty Ltd.), before incubating in a 450-GC oven (Bruker Daltonics), pre-heated to 75°C, for 60 minutes. The samples were mixed on low using a vortex, IKA MS1 Works (Crown Scientific Pty Ltd.), at 15 minute intervals during this 60 minutes incubation period.

The vials were removed from the GC oven and set aside to rest for two hours before analysis by GC-MS.

The samples were analysed in random order including a 20 minute methanol blank between all samples and 60 minute methanol blank every four injections. QC samples were used to equilibrate the system ahead of sample analysis and analysed within the sample batch every fifth sample.

2.2.5.1.1 Milk volume optimisation for derivatisation

A series of 5 milk samples (from each of the sampling groups) were prepared in duplicate to determine the optimal volume for milk analysis by GC-MS; one human control, one nipple pain without trauma, one nipple pain with trauma, one bovine control and one bovine mastitis. Two 600 µl aliquots of each sample was used and prepared according to the protein precipitation method in 2.2.5.1. The resultant supernatant (1,600 µl) was dispensed into a 2 ml centrifuge tube and divided into two sets of four volumes 75, 150, 225 and 300 µl, sample volumes containing an equivalent volume of 25, 50, 75 and 100 µl of milk, respectively. Internal standards of

65 µl of 500 µg/ml $^{13}C_6$ Sorbitol (Sigma Aldrich Pty. Ltd.) and 45 µl of 10 µg/ ml 2-aminoanthracene (Sigma Aldrich Pty. Ltd.) (in water) plus 1,455 µl of water (Thermo Scientific Australia Pty. Ltd.) was added to each sample. All samples were frozen on dry ice and dried by lyophilisation. One of each duplicate set of four was stored at -80ºC for future studies.

Samples were redissolved and fractioned by SPE followed by derivatisation according to the methods in 2.2.5.1 Milk samples were analysed in random order using a Shimadzu QP2010 Ultra GC-MS (Shimadzu Corporation).

The optimal sample volume was determined with compromise between the detected signal (peak area) of each metabolite; particularly those present in smaller concentrations, instrument capability (efficiency and required preventive maintenance) and the ability to dissolve the dried metabolite extracts; with data reproducibility being the major deciding factor. The optimal equivalent volume of milk required was determined to be 50 µl; this equivalent volume was used for all subsequent analyses.

2.2.5.1.2 Derivatisation temperature optimisation

Metabolite reference standard mixes (mix containing 30 metabolites, Table 2.5) were derivatised following the derivatisation method described in section 2.2.5.1, with the exception that after the addition of MSTFA (Sigma Aldrich Pty. Ltd.) each were incubated at one of five temperatures. The MSTFA derivatisation was assessed at the five temperature increments, 37°C, 45°C, 60°C, 75°C and 90°C, using a 450-GC oven (Bruker Daltonics). Two controls were also included for the 37ºC and 60ºC reaction

temperatures, which additionally varied from the described methods in that they were not transferred to glass vials before MSTFA addition. These two sets were heated in an Eppendorf Thermomixer® comfort (Crown Scientific Pty Ltd.) within 2 ml tubes for comparison to already established methods. Samples incubated within vials were sealed by crimp cap after the addition of MSTFA and gently mixed using a vortex, IKA MS1 Works (Crown Scientific Pty Ltd.) at 15 minute intervals during incubation. All were prepared in triplicate.

The vials were removed from the GC oven and Thermomixer and set aside to rest for two hours before analysis using a Shimadzu QP2010 Ultra GC-MS (Shimadzu Corporation).

Each reference metabolite peak was de-convoluted from the total ion chromatogram (TIC) and peak area(s) calculated using AnalyzerPro 2.7.0.0 (SpectralWorks Ltd.). The standard deviation was calculated using the measured technical replicates, which was plotted for each metabolite at each temperature and heating method. The optimal temperature was determined by the reproducibility of the triplicate measurements for each temperature. The optimal MSTFA derivatisation temperature was determined to be 75 °C; this temperature was used for all subsequent analyses.

2.2.5.2 Preparation of the final sample set (using the optimised methodology)

Milk samples were dispensed in 500 µl volumes into 2 ml centrifuge tubes. To each sample was added 26 µl of 500 µg/ml $^{13}C_6$ Sorbitol and 60 µl of 10 µg/ml 2-aminoanthracene (each in LC grade water), in a final volume of 600 µl (by the

addition of LC-MS grade water). Each were stored at -80°C and thawed on ice prior to preparation.

Milk samples were prepared by protein precipitation and fractioned by SPE to isolate the polar metabolites according to methods in 2.2.5.1, with a final dried down volume equivalent to 50 µl of milk. Dried samples were derivatised according to the method in 2.2.5.1. prior to analysis using a Shimadzu QP2010 Ultra GC-MS (Shimadzu Corporation).

For the purposes of quality control (QC), a second set of 500 µl aliquots was removed from the -80°C freezer and pooled by combining 175 µl from each of the 42 samples in a 10 ml falcon tube, followed by gently mixing using a vortex, IKA MS1 Works (Crown Scientific Pty Ltd.). The pooled QC milk was dispensed and prepared randomised among the samples described above.

2.2.5.3 Instrumentation and data acquisition

GC-MS analysis was carried out using a Shimadzu QP2010 Ultra GC-MS (Shimadzu Corporation, Kyoto, Japan) injected with 1µl of derivatised milk metabolites. The GC-MS was fitted with a FactorFour VF-5ms capillary column (30m x 0.25mm x 0.25µm + 10m EZ-Guard; Agilent, Santa Clara USA). The injection inlet temperature was set to 230 °C, with an interface temperature of 300 °C, and an ion source temperature of 230 °C. Helium was selected as the carrier gas and set to flow between 0.8 and 1.0 ml min^{-1}. Prior to sample injection the inlet pressure was adjusted to elute mannitol (6-TMS) at 30.6 minutes. The temperature gradient commenced at an initial temperature of 70°C, increasing at 1°C per minute for 5 minutes, then changing to an

oven ramp rate of 5.6 °C min^{-1} with a 10 minute hold at the final maximum temperature of 320°C. Ionisation occurred by electron ionization (EI) at 70 eV. The mass spectrometer was operated in scan mode in the range m/z 40 – 600, at a scan rate of 5,600 amu sec^{-1}. For selected ion monitoring (SIM), ions were scanned at 0.38 second intervals.

2.2.5.4 Data analysis

GC-MSsolutions 2.61 (Shimadzu Corporation, Kyoto, Japan) was used to acquire and analyse the data post GC-MS metabolomic analysis. AnalyzerPro 2.7.0.0 (SpectralWorks Ltd.) was used for deconvolution. Appropriate qualifier and quantifier ions were determined from the MS and the full scan spectra searched again against the in house metabolite library and the already predetermined database (a list of tentative metabolite identifications compiled during method optimisation of the sample volume) of metabolite identities, for relative quantitation using the peak area of the analyte.

2.2.5.5 Data processing and interpretation

For the method optimisations, analyte peak areas were interrogated using JMP 8.0.2 (SAS Institute Inc.). For the metabolomics analyses, peak areas were normalised to the internal standard, $^{13}C_6$ sorbitol, and scaled to the median intensity (De Livera et al., 2012), range scaled and log transformed ($\log_{10}(x + 1)$ using The Unscrambler (Camo) software. Principal component analysis (PCA) was used to model the transformed data and PCA correlation loadings calculated using The Unscrambler (Camo).