European Innovation Partnership (EIP) Wales

Final Project Evaluation

Night Milk: Assessing the reliability and economic benefit

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Cronfa Amaethyddol Ewrop ar gyfer Datblygu Gwledig: Ewrop yn Buddsoddi mewn Ardaloedd Gwledig
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Section 1: INTRODUCTION

EIP is part of the Co-operation and Supply Chain Development Scheme (CSCDS) delivering under Measure 16 (Article 35 of Regulation (EU) 1305/2013). The CSCDS is an important element of the Welsh Government Rural Communities - Rural Development Programme 2014-2020. The EIP delivers under sub Measure 16.1 of the Welsh Government Rural Communities - Rural Development Programme 2014-2020. Menter a Busnes has been awarded the contract for delivering EIP in Wales on behalf of the Welsh Government.

The European Innovation Partnership on Agricultural Sustainability and Productivity (EIP-AGRI) was launched by the European Union in 2012 to contribute to the Union's 'Europe 2020' strategy for smart, sustainable and inclusive growth. This strategy sets the strengthening of research and innovation as one of its five main objectives and supports a new interactive approach to innovation: EIP.

EIP aims to contribute to the steady supply of food, feed, biomaterials and to the sustainable management of the essential natural resources; on which farming and forestry depend, working in harmony with the environment. To achieve this aim, EIP brings together innovation actors (farmers, advisors, researchers, businesses, non-governmental organisation (NGOs), etc.) and helps to build bridges between research and practice through Operational Groups (OG's). Pooling expertise and resources by bringing groups of people from different practical and scientific backgrounds together to tackle specific challenges, and trial new approaches which will be of value to others in the agricultural or forestry industry. Group projects are considered and appraised in line with the aims of the EIP listed in Article 55 of Regulation (EU) No 1305/2013, fostering a competitive and sustainable agriculture and forestry sector. Individual projects are managed and supported by 'Innovation Brokers', who are funded (separately and in addition to the EIP programme) though Farming Connect.

The project being reviewed, is part of the European Innovation Partnership programme and has received funding through the European Agricultural Fund for Rural Development. In Wales the implementation of the EIP programme is managed by Menter a Busnes who also manage the Farming Connect programme.

Innovation is seen as a new idea that proves successful in practice. It may be technological, but also non-technological, organisational or social; and may be based on new but also on traditional practices in a new geographical or environmental context. The new idea can be a new product, practice, service, production process or a new way of organising things, etc. A new idea turns into an innovation only if it is widely adopted and proves its usefulness in practice.

One of the main focus points of approved projects are in areas that include technical solutions to increasing productivity or resource efficiency.

Section 2: PROJECT DESCRIPTION

Night Milk to assess the reliability and economic benefit of Melatonin

Milk has long been known and used to promote sleep. The sleep-promoting effect of milk has been attributed to its rich store of sleep-promoting components (dela Peña et al., 2015). Melatonin is the hormone that helps control sleep and wake cycles and is produced naturally in response to darkness.

Melatonin is a hormone that helps control sleep and wake cycles and is produced naturally in response to darkness (Hardeland et al., 2006). It can be found in a variety or organisms and in animals it is a hormone secreted by the pineal gland in the brain.

In relation to cows, the Melatonin hormone is produced in the cow's pineal gland and when light hits a cow's eye, it signals the cow's body to produce less melatonin. When it is dark, melatonin is produced. Cows have an internal clock that is set by melatonin production. This internal clock affects the production of other hormones that impact milk production. For example, long-day lighting increases the production of IGF-I, the more IGF-I produced by the cow results in increase milk production. Melatonin is found in bovine milk at concentrations between 5-25 pg mL-1 (Jouan et al., 2006). Raw milk contains the highest melatonin concentrations, and processing to extend the life of milk has a negative effect on melatonin content (apart from 'night-time milk powder', marketed as melatonin rich) and it's concentration appears to vary between individual cows but is not affected by fat/protein ratio, milk yield, somatic cell score or stage of lactation (Schaper et al., 2015).

Much research has been undertaken in the field of melatonin – its use to optimise sleep or improve sleep quality, to prevent jet lag and to improve insomnia. Human melatonin production decreases as a person ages, leading to insomnia and changes in circadian rhythmicity. A study undertaken in 2005 showed a positive effect of melatonin, given in the form of a night-time milk drink, on the sleep quality of elderly institutionalised subjects (Valtonen et al, 2005). Another study in 1998 also demonstrated that melatonin has a beneficial sleep-inducing effect in elderly melatonin-deficient insomniacs and in children with sleep disorders (Luboshizsky and Lavie, 1998).

It has alss been established that the compound Tryptophan is a pre-cursor for Serotonin and Melatonin, therefore is converted into these compounds in the body. Serotonin (5-HT) is a neurotransmitter that is associated with mood. Normal serotonin production can help maintain a balanced mood, promote relaxation, and help improve memory, Tryptophan is also found at high levels in cow's milk (Dairy Nutrition, n.d.) and is consequently an amino acid that induces sleep.

A literature search undertaken by both Dr. Ruth Wonfor of IBERS and Janet Holmes - Zero2Five (Cardiff Metropolitan University) prior to the submission of the EIP application for funding support, found that scientific research papers had been published to indicate that there is a notable benefit from Melatonin enriched milk and that there was potential for further research systems to increase the concentration of melatonin in cows' milk.

Project Rationale

With conventional milk supply affected by erratic markets and volatile milk prices, the need to identify point of difference and unique selling points (USP'S) is ever more so prevalent within the dairy sector. Milk supply with a rich source of any beneficial naturally produced compoundwould offer such USP and command increase revenue for farmers. Despite the inconclusive research undertaken to date, the EU Register of Nutrition and Health claims holds two authorised claims that can be made on Melatonin; these claims were authorised in 2011 and 2010, respectively (EFSA, 2011; 2010). The information provided below is taken directly from the EU Register of Nutrition and Health claims:

1) Melatonin contributes to the reduction of time taken to fall asleep. The claim may be used only for food which contains 1mg of melatonin per quantified portion. In order to bear the claim, information shall be given to the consumer that the beneficial effect is obtained by consuming 1mg of melatonin close to bedtime.

2) Melatonin contributes to the alleviation of subjected feelings of jet lag. The claim may be used only for food which contains at least 0.5mg of melatonin per quantified portion. In order to bear the claim, information shall be given to the consumer that the beneficial effect is obtained with a minimum intake of 0.5mg to be taken close to bedtime on the first day of travel and on the following few days after arrival at the destination.

Project Participants/Actors

Lead Farmer:	Rhys Loucher – Ty Tanglwyst Farm, Pyle, Bridgend, CF33 4SA
Participant Farmer:	Philip Anthony - Haregrove Farm, Bridgend, CF32 0NR
EIP Project Lead:	Neil Blackburn, Kite Consulting, The Dairy Lodge, Dunston Business Village, Dunston, Staffordshire, ST18 9AB

Innovation Broker: Russell Thomas, c/o Kite Consulting,

Objectives

This project has been led by Kite Consulting with Lead Farmer Participant - Rhys Lougher. It has been managed by 'Innovation Broker' Russell Thomas of Kite Consulting.

The project sought to assess the levels of melatonin on segregated milk from Ty Tanglwyst Farm via laboratory testing of samples. The milk to be segregated will be from the early hours of the morning. The project aims were to separate the 'night milk' and sample it for Melatonin content by commissioning recognised laboratories to develop testing methodologies to calculate accurate levels of melatonin content and to then carry out the testing.

A second farm provided the 'control' where they would monitor the differences between their am and pm milking and which were then to be compared against the results being recorded at Ty Tanglwyst.

The project in its purest terms was essentially involved in testing samples of milk for melatonin levels, in order to determine whether there was a difference in levels between milk produced during the day and milk produced during the night. Seasonal variations (summer / winter lighting) were expected to be taken into consideration as would environmental factors such as Housing and light regulation. Other factors such as feed (e.g. amount of clover); increasing dietary input of tryptophan (the precursor for melatonin) which is found in most foods high in protein; and could also effect production of melatonin; and the effects of this were likely to have to be considered.

Timescale

The project was proposed to run for up to 13 months from 10th April 2018 to 31 May 2019. However delays in the application, EIP funding approval and commissioning of laboratories suspended the start until September 2018

Project Location

The project work involving sample collection was carried out on 2 grass based farms in the Bridgend area of Mid Glamorgan. Melatonin testing would require recognised off-site approved laboratories.

Project activities

Melatonin levels in milk (amongst other liquids) have been measured using a variety of scientific methods, most of which were found to be inaccurate and insufficiently standardised (Garcia-Parrilla *et al*., 2009). However, a study published in 2014, developed a new analytical method to quantify the amount of melatonin in milk which claims to be more specific and selective, giving a lower detection limit compared to other techniques. It uses Liquid Chromatography-Mass Spectrometry (LCMS) (Karunanithi *et al*., 2014).

With this in mind, the project was to consider the following areas:
1. Test samples of milk for melatonin levels, in order to determine whether there is a difference in levels between milk produced during the day and milk produced during the night. Seasonal variations (summer / winter lighting) need to be taken into consideration. Other factors such as feed (e.g. amount of clover) may also effect production.
2. If levels are >1mg per quantified portion (suggested portion 250ml) a claim can be made.
3. If levels are <1mg per quantified portion, the product could still be called ‘night milk’ (i.e. milk obtained from cows during the night), however, a claim cannot be made on the melatonin content or its potential beneficial effect on sleep.

In terms of quantifying melatonin concentrations within milk within this project and having already established that liquid chromatography-mass spectrometry (LC-MS), has been successfully developed for quantification of melatonin levels. Specialist expertise was required and consequently the project adhered to the Welsh Government technical guidance notes on public procurement which indicated that the project must demonstrate that there has been fair and open practices by using a competitive tendering exercise for all goods or services that are included in the project for which there is the intension to claim grant support. As a result, the melatonin testing element of this project was advertised and put out to tender via Sell2Wales in compliance; as set out; within the following table.

The thresholds and the requirements for a competitive tendering exercise are shown in the table below:

Final Value of the goods or services purchased	Competitive Tendering Requirement	Action
£0 - £4,999	One written quote	It is recommended to use suppliers that are registered on www.sell2wales.gov.wales
£5,000 – £24,999	Three written quotes	It is recommended to use suppliers that are registered on www.sell2wales.gov.wales
£25,000 and above	Full and open competition	Requirements **must be published** through www.sell2wales.gov.wales Quotes may be sought by direct reference to suppliers

Although LC-MS is the prefered method, contractors weare encouraged to present alternative testing methodology. This was verified via consultation with the bidding contractors.

The successful contractor appointed to test milk samples; via the preferred liquid chromatography-mass spectrometry (LC-MS); was **Fera Science Limited ("Fera"), National Agri-Food Innovation Campus, Sand Hutton, York, YO41 1LZ, United Kingdom**

Section 3: METHODOLOGY

This project sought to assess the levels of melatonin on segregated (separately processed) milk from Ty Tanglwyst Farm via laboratory analysis of samples. The milk to be segregated was taken during milking in the early hours of the morning ('NightMilk'). The project aimed to separate the 'Night Milk' and analyse it for melatonin content.

Fera Science Ltd (Fera) was commissioned to develop testing methodologies to calculate accurate concentrations of melatonin in milk and to undertake the analyses of the test samples. A second farm provided the 'control' milk samples, providing milk samples from both day and night milking. These were then compared to the results from the Ty Tanglwyst samples; of particular interest were results for the 'Night Milk' samples.

The project sought to determine whether there is a difference in melatonin concentrations in milk produced during the day and milk produced during the night.
The work at Fera, firstly involved development / optimisation of analytical methods before validating the methodology for the accurate determination of melatonin at trace concentrations (parts per billion) in milk.

A stability study was also undertaken on raw and pasteurised milk, fortified with melatonin.
Alongside conventional targeted analysis for melatonin a further screening method was employed to look for raised concentrations of other compounds relating to the metabolism of tryptophan in the samples.

SAMPLE DESCRIPTION

A. Stability Study
To undertake the method validation and the stability study, 2 bulk reference samples were purchased from local suppliers to the laboratory, a 1L raw milk sample from Cow Corner,

Thirsk, N.Yorkshire (LIMS no: S19-034277) and a 1L whole milk (LIMS no: S18-039488) sample from Sainsburys, Pocklington, E.Yorkshire.

B. Test Samples

Samples were received from Rhys Lougher-Ty Tanglwyst at 5 sampling time points between September 2018 and April 2019. On arrival sample containers were assessed for damage before the samples were assigned a unique laboratory information management system (LIMS) number. Details are provided in Table 1 (note, where milking start time is before midnight, this is the previous day).

Table 1. Samples and details provided by Rhys Lougher

Supplier sample number	Fera LIMS number	Date Collected	Time Collected	Farm	Milking start time	Raw / Pasteurised
1	Not assigned, this sample was damaged in transit to Fera					
2	S18-045307	24/9/18	00.15	Ty Tanglwyst	23.45	Pasteurised
3	S18-045308	24/9/18	00.15	Ty Tanglwyst	23.45	Raw
4	S18-045309	24/9/18	00.15	Ty Tanglwyst	23.45	Raw
5	S18-045310	24/9/18	9.15	Ty Tanglwyst	7.45	Raw
6	S18-045311	24/9/18	9.15	Ty Tanglwyst	7.45	Raw
7	S18-045312	24/9/18	15.15	Haregrove	15.00	Raw
8	S18-045313	24/9/18	15.15	Haregrove	15.00	Raw
9	S18-045314	24/9/18	23.00	Haregrove	21.30	Raw
10	S18-045315	24/9/18	23.00	Haregrove	21.30	Raw
11	S18-054465	31/10/18	00.15	Ty Tanglwyst	23.45	Pasteurised
12	S18-054466	31/10/18	00.15	Ty Tanglwyst	23.45	Pasteurised
13	S18-054467	31/10/18	00.15	Ty Tanglwyst	23.45	Raw
14	S18-054468	31/10/18	00.15	Ty Tanglwyst	23.45	Raw
15	S18-054469	31/10/18	9.00	Ty Tanglwyst	7.45	Raw
16	S18-054470	31/10/18	9.00	Ty Tanglwyst	7.45	Raw
17	S18-054471	31/10/18	22.00	Haregrove	21.30	Raw
18	S18-054472	31/10/18	22.00	Haregrove	21.30	Raw
19	S18-054473	31/10/18	8.00	Haregrove	6.00	Raw
20	S18-054474	31/10/18	8.00	Haregrove	6.00	Raw
21	S18-059395	11/12/18	23.50	Ty Tanglwyst	23.45	Pasteurised
22	S18-059396	11/12/18	23.50	Ty Tanglwyst	23.45	Pasteurised
23	S18-059397	11/12/18	23.50	Ty Tanglwyst	23.45	Raw

Supplier sample number	Fer a LIM S number	Date Collected	Time Collected	Farm	Mliki ng s tart time	**Raw** / Pasteuri sed
2A	S18-059398	11/12/18	23.SO	TyT8 1W'f"I	23AS	... N
26	$ 1&-059399	11/12/18	9.15	Ty Tilll"QIW)1,1	7.45	... N
26	S18-059400	11/12/18	9.15	TyTargl.,.-,.,,,1	7.45	... N
V	S18-059401	11/12/18	7.15	Har	6.30	... N
26	S18-059402	11/12/18	7.15	Haregrme	6.30	... N
29	$ 18-059403	11/12/18	**22.00**	**Haregrme**	21. 30	... N
30	S18-059404	11/12/18	22.00	Har	21.30	... N
31	S19-011911	1712/19	22.00	Haregro,.e	21.30	... N
32	S19-011912	17/2/19	22.00	Haregro'l,8	21. 30	... N
33	$19-011913	1&'2/19	7.15	**Haregrme**	6 . 30	... N
34	S 19-0 1191 4	1812/19	7.15	Har	6.30	... N
3S	S19-011915	17/2/19	23.SS	T'(Ta rgl'N';SI	23. 35	... N
36	$19-011916	1 712/19	23.SS	TyTa l I	2335	... N
37	$19-01971	172/19	23.SS	TyTargl.,.-,.,,,1	23. 35	... N
36	S19-011918	17/2/19	23.SS	T'(Ta rgl'N';SI	23. 35	... N
39	S19-011919	1712/19	23.55	T'(Ta r,glwy31	23.35	... N
•0	S19-011920	17/2/19	23.55	TyT8 1W'f"I	23AS	... N
• 1	$19-011921	1 712/19	23.SS	Ty Tilll"QIW)1,1	23.45	... N
	S19-011922	17/2/19	23.SS	T'(Ta r,glwy31	23.45	... N
•3	S19-011923	17/2/19	23.55	TyT8 1W'f"I	23AS	... N
0 0	$ 19-0 1192 4	1 712/19	23.S S	TyTa l I	2345	... N
•S	$19-01952	1&'2/19	**6.00**	TyTargl.,.-,.,,,1	7.4 5	... N
• &	S19-011926	1812/19	**6 .00**	T'(Ta rgl'N';SI	7.4 -5	... N
• 1	S19-011927	1812/19	**6.00**	T'(Ta rgl'N';SI	7.4S	... N
,&	$19-011928	1&'2/19	**6.00**	TyTargl.,.-,.,,,1	7.4 5	... N
• 9	S19-011929	1812/19	**6 .00**	TyTarQIW)1,1	7.4 -5	... N
SO	S19-011930	1812/19	**6 .00**	T'(Ta rgl'N';SI	7.4 -5	... N
51	S19-011931	1812/19	**6.00**	TyT8 1W'f"I	7.45	... N
52	$19-011932	1&'2/19	**6.00**	TyT8 1W'f"I	7.45	... N

Supplier sample number	Fera LIMS number	Date Collected	Time Collected	Farm	Milking start time	Raw / Pasteurised
53	S19-011933	18/2/19	1.00	Ty Tanglwyst	23.45	Raw
54	S19-011934	18/2/19	1.00	Ty Tanglwyst	23.45	Raw
55	S19-019132	25/4/19	1.00	Ty Tanglwyst	23.45	Raw
56	S19-019133	25/4/19	1.00	Ty Tanglwyst	23.45	Raw
57	S19-019134	25/4/19	1.00	Ty Tanglwyst	23.45	Raw
58	S19-019135	25/4/19	1.00	Ty Tanglwyst	23.45	Raw
59	S19-019136	25/4/19	1.00	Ty Tanglwyst	23.45	Raw
60	S19-019137	25/4/19	1.00	Ty Tanglwyst	23.45	Raw
61	S19-019138	25/4/19	1.00	Ty Tanglwyst	23.45	Raw
62	S19-019139	25/4/19	1.00	Ty Tanglwyst	23.45	Raw
63	S19-019140	25/4/19	1.00	Ty Tanglwyst	23.45	Raw
64	S19-019141	25/4/19	1.00	Ty Tanglwyst	23.45	Raw
65	S19-019142	25/4/19	1.00	Ty Tanglwyst	23.45	Raw
66	S19-019143	25/4/19	1.00	Ty Tanglwyst	23.45	Raw
67	S19-019144	25/4/19	7.30	Ty Tanglwyst	7.00	Raw
68	S19-019145	25/4/19	7.30	Ty Tanglwyst	7.00	Raw
69	S19-019146	25/4/19	7.30	Ty Tanglwyst	7.00	Raw
70	S19-019147	25/4/19	7.30	Ty Tanglwyst	7.00	Raw
71	S19-019148	25/4/19	7.30	Ty Tanglwyst	7.00	Raw
72	S19-019149	25/4/19	7.30	Ty Tanglwyst	7.00	Raw
73	S19-019150	25/4/19	7.30	Ty Tanglwyst	7.00	Raw
74	S19-019151	25/4/19	7.30	Ty Tanglwyst	7.00	Raw
75	S19-019152	25/4/19	7.30	Ty Tanglwyst	7.00	Raw
76	S19-019153	25/4/19	7.30	Ty Tanglwyst	7.00	Raw
77	S19-019154	25/4/19	7.30	Ty Tanglwyst	7.00	Raw
78	S19-019155	25/4/19	7.30	Ty Tanglwyst	7.00	Raw

Samples 66 and 78 were described as “bulk samples” which were samples consisting of all the milk collected on that date / time.

VALIDATION

Method validation:

Before acceptance of samples, method validation was undertaken to ensure the method was accurate, precise and limits of detection were fit for purpose.

Fortified (over spiked) milk samples were prepared in pasteurised milk. Six replicates at 0.01 and 0.1 µg/mL melatonin concentrations were prepared before extraction and determination by Liquid Chromatography-tandem quadrupole Mass Spectrometry (LC-MS/MS). Quantification was undertaken using a prepared calibration range from an analytical standard in methanol between 0.005 and 0.5 µg/mL. An internal standard (melatonin-d4) was added to all the fortified samples and calibrants to correct for any matrix effects in the quantification calculation.

Materials:

The melatonin reference and deuterated standards were purchased from Sigma-Aldrich and Cayman Chemicals respectively.

Stability study:

To evaluate the stability of melatonin in raw and pasteurised milk, a study was undertaken to measure melatonin concentrations over a 3-week period, in fortified samples stored at 2 different storage conditions: (i) in the fridge (~4°C) and (ii) in the freezer (~-20°C).

To ensure the absence of melatonin (< 0.005 µg/mL), the bulk raw and pasteurised milk samples were analysed before preparation of any fortified material (and classed as 'blank material').

Into 1L each of raw and pasteurised milk 1 mL of 1 mg/mL melatonin reference standard was added a to give fortified samples at 1 µg/mL for this study. These 1 L fortified bulk materials were thoroughly stirred with a magnetic flea for approximately 1 hour, before aliquots (~ 2 mL) were transferred to amber glass vials and stored at either 4°C or -20°C.

Samples were analysed in triplicate at the following time points: Day 0, Day 6, Day 10, Day 16 and Day 20.

An analytical solvent standard (melatonin in methanol) at 0.1 µg/mL was prepared, stored at 4°C and analysed at the following timepoints: Day 0, Day 1, Day 6, Day 10, Day 16 and Day 20.

SAMPLE EXTRACTION

All fortified samples of the method validation and stability study, and test samples received from Rhys Lougher, were prepared for analysis as follows:

The methodology was based on Karunanithi, et, al[1]. All samples had the internal standard melatonin-d4 added to them at a concentration of 0.1 µg/mL before extraction.

Briefly, 1 ml of sample was added to a 5 mL chem elut solid phase extraction cartridge (Agilent). The cartridge was eluted with 15 mL of dichloromethane, evaporated to dryness at room temperature under nitrogen before reconstitution with methanol. All samples were then filtered through 0.22 µm PVDF filter before analysis by LC-MS/MS.

1 Karunanithi, D., Radhakrishna, A., Sivaraman, KP. And Biju, VM (2013). Quantitative determination ofmmelatonin in milk by LC-MS/MS. Journal of Food Science and Technology, 51(4):805-812

LC-MS/MS

LC analysis was performed on an Acquity UPLC system from Waters Corporation. The column used was a Poroshell 120 (EC) C18 100 x 2.1mm, 2.7 µm (Agilent). Mobile phases were 5 mM ammonium acetate in water (mobile phase A, MPA) and methanol (mobile phase B, MPB). Gradient applied was 98% MPA for 2 minutes before increasing to 98% MPB over 6 minutes. This was held for 2 minutes before reverting to 98% MPA and held for 2 minutes. Injection volume was 3 µL, flow rate was 0.4 mL/min and column temperature was 40°C. The MS used was a Quattro Premier XE (Waters Corporation) set with an acquisition window between 4 and 6 minutes inpositive ion mode. Data acquisition conditions are summarised in Table 2.

Data was evaluated and melatonin quantified using Masslynx 4.1 (Waters Corporation).

The limit of quantification for this analysis was 0.005 µg/mL, with a limit of detection estimated to be approximately half of this (0.0025 µg/mL) based on the signal: noise of the bottom calibrant. All quantification was corrected using the deuterated internal standard response.

Table 2. Summary of MS/MS acquisition conditions for melatonin and melatonin-d4.

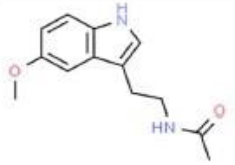
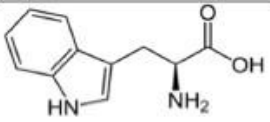
Compound	MS/MS transition	Cone Voltage	Collision Voltage	Dwell time (ms)	Retention time (min)
Melatonin	233 > 130	27	45	50	5.5
	233 > 159	27	27	50	
	233 > 174	27	15	50	
Melatonin-d_4	237 > 178	27	15	50	5.5

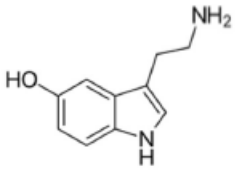
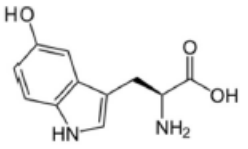
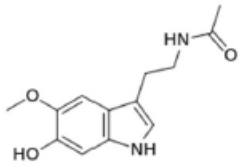
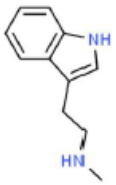
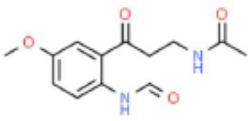
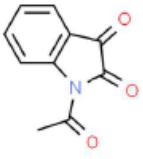
SCREENING ANALYSIS

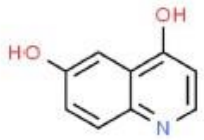
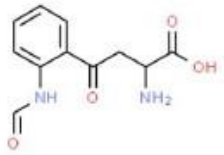
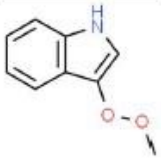
Screening details:

Further to the targeted analysis for melatonin, the first 19 samples (samples 2 – 20 from Table 1) were screened for other compounds relating to tryptophan metabolism. This was using the same sample extract prepared for the main targeted study, before analysis by Liquid Chromatography - High Resolution Mass Spectrometry (LC-HRMS). A summary of all 11 compounds screened by this method is given in Table 3.

Table 3. Summary of compounds included in the HRMS screening methodology

Compound	Formula	Mass	Structure
Melatonin	$C_{13}H_{16}N_2O_2$	232.1212	
Tryptophan	$C_{11}H_{12}N_2O_2$	204.0899	

Compound	Formula	Mass	Structure
Seroton in	C10H 12N 2O	176 .0950	
5-Hydroxytryptophan	C11H 12N2O3	**220.0848**	
6-Hydroxymelatonin	C13H 15N 2O3	248 .1161	
N-Methyl tryptamine	C11H 14N 2	174 . 1 157	
Formyl-n-acetyl-5 methoxykynurenamine	C 13H 15N 2O4	264 . 1 110	
N-Acetylisatin	C10H 1NO 3	189 .0426	

Compound	Formula	Mass	Structure
4,6-Dihydroxyquinoline	$C_9H_7NO_2$	161.0477	
N-Formylkynurenine	$C_{11}H_{12}N_2O_4$	236.0797	
3-Methyldioxyindole	$C_9H_9NO_2$	163.0633	

LC-HRMS

LC analysis was performed on an Accela High Speed LC system from Thermo Fisher Scientific. The column used was an ACE 3Q 150x3mm, 3µm (Advanced Chromatography Technologies). Mobile phases were 0.1% formic acid in water (mobile phase A, MPA) and 0.1% formic acid in acetonitrile (mobile phase B, MPB). Gradient applied was 100% MPA for 5 minutes before increasing to 100% MPB over 15 minutes. This was held for 10 minutes before reverting to 100% MPA and held for 2 minutes. Injection volume was 10 µL, flow rate was 0.4 ml/min and column temperature was 25°C. The MS used was a Thermo Exactive (Thermo Fisher Scientific) set at 50,000 resolution FWHM @ 200 m/z with an acquisition speed of 2Hz.

Data was acquired in two separate batches to cover both positive and negative ionisation modes for greater compound coverage. Samples were analysed in a random order derived from www.random.org.

Data was evaluated using Xcalibur qual software (Thermo Fisher Scientific).

Section 4: RESULTS

METHOD VALIDATION

The method was deemed to be suitable for precision and accuracy when evaluating the fortified samples at two concentrations. A summary of this data is presented in Table 4. Figures 1 and 2 show example chromatography in a sample fortified with melatonin at 0.01 and 0.1 µg/ml, Figure 3 shows example chromatography for a solvent standard at 0.005 µg/ml and Figure 4 shows an example calibration curve used for quantification.

Table 4. Summary of method validation data for melatonin

	Fortified concentrations in pasteurised milk (µg/mL)	
	0.01	**0.1**
Mean concentration [µg/mL]	0.0103	0.1008
Precision [CV%]	1.6	2.0
Accuracy [%]	103	101
Number of replicates	6	6

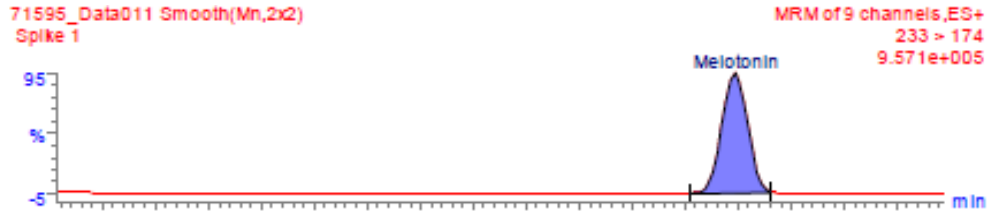


Figure 1. Example melatonin chromatography (233>174), in a fortified pasteurised milk sample at 0.01 µg/mL.

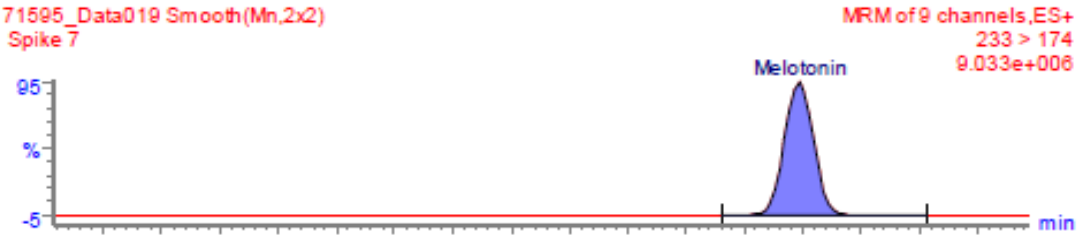


Figure 2. Example melatonin chromatography (233>174), in a fortified pasteurised milk sample at 0.1 µg/mL.

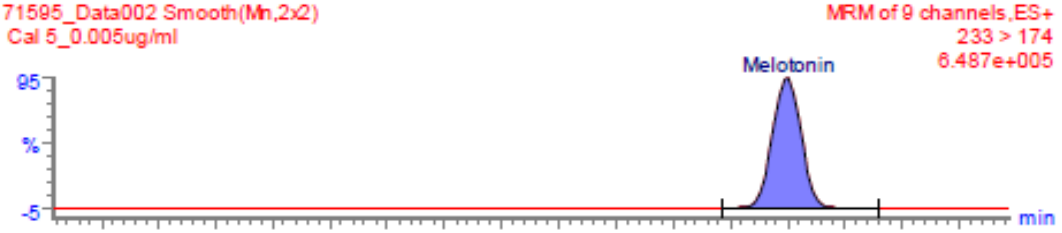


Figure 3. Example melatonin chromatography (233>174), in a solvent standard at 0.005 µg/mL.

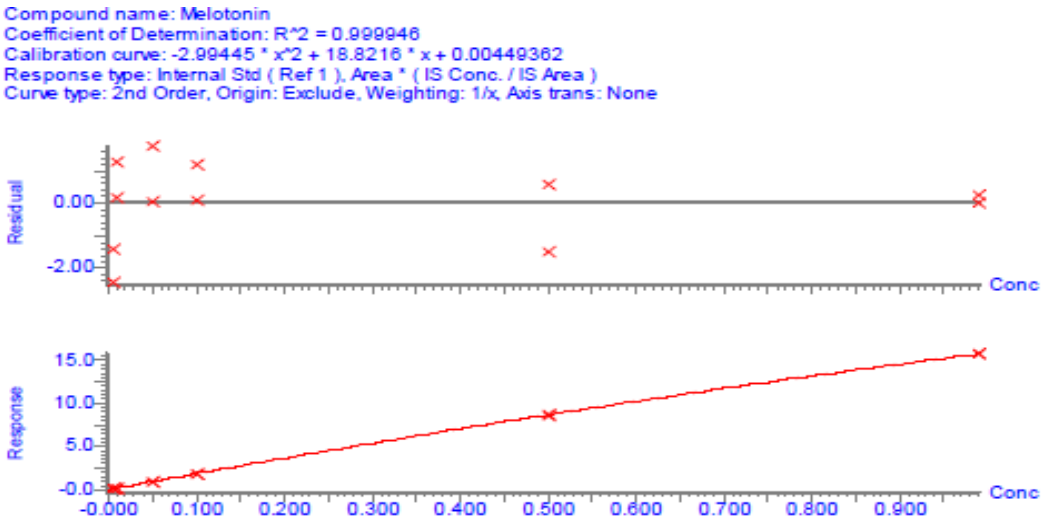


Figure 4. Example calibration curve of solvent standards of melatonin between 0.005 and 1 µg/mL.

Stability Study

Both, the pasteurised and the raw milk samples fortified at 1 µg/ml and stored at both conditions (4°C and - 20°C) showed a stable concentration of melatonin across the 20 day period studied. Figures 5 to 9 show the plot of melatonin concentrations across each of the analysis time points for both milk types and storage conditions, plus a plot for the melatonin solvent standard also evaluated for stability.

This provided confidence that samples could be stored chilled or frozen for at least 3 weeks before analysis without risking a breakdown of melatonin and therefore a decrease in concentration.

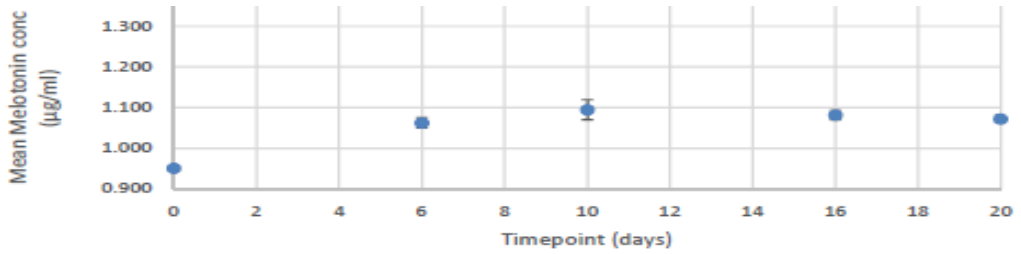


Figure 5. Concentrations of melatonin in a fortified pasteurised milk sample (1 µg/mL) stored at 4°C over a time period of 20 days.

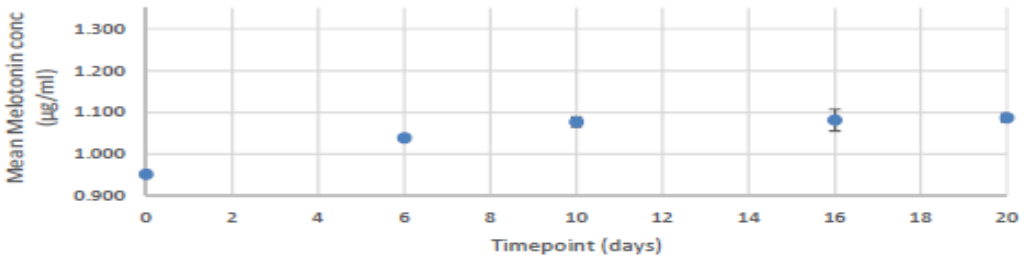


Figure 6. Concentrations of melatonin in a fortified pasteurised milk sample (1 µg/ml) stored at -20°C over a time period of 20 days.

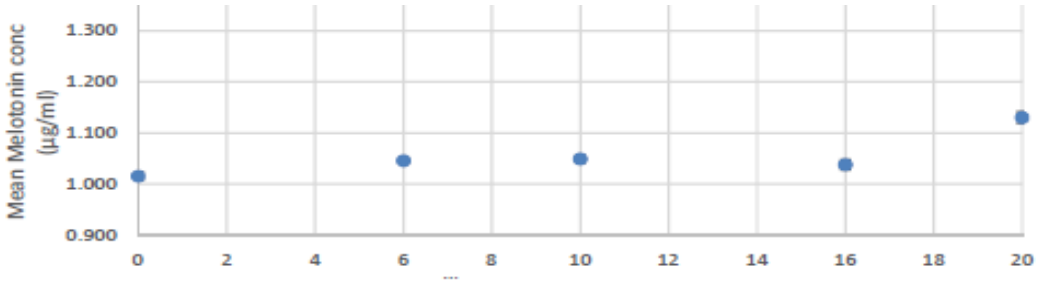


Figure 7. Concentrations of melatonin in a fortified raw milk sample (1 µg/ml) stored at 4°C over a time period of 20 days.

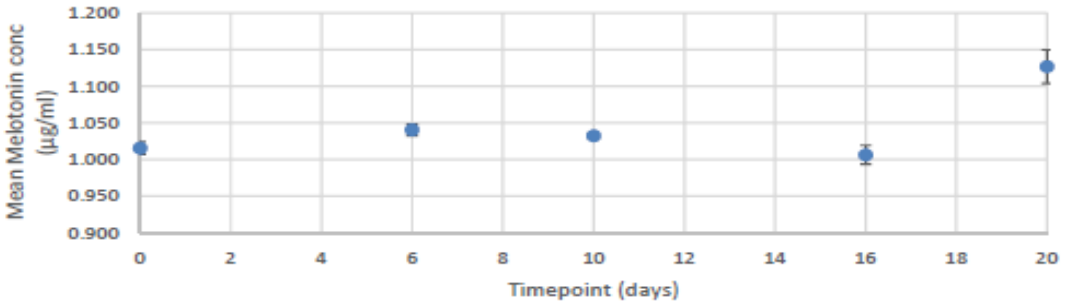


Figure 8. Concentrations of melatonin in a fortified raw milk sample (1 µg/ml) stored at -20°C over a time period of 20 days.

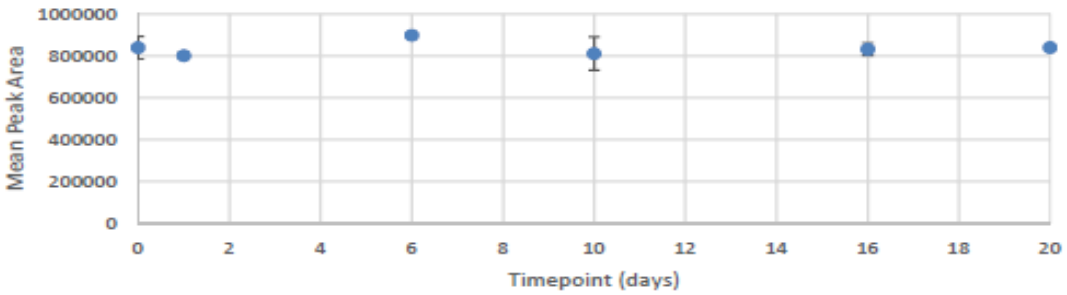


Figure 9. Response (peak area) of melatonin in a solvent standard at 0.1 µg/ml stored at 4°C over a time period of 20 days.

TEST SAMPLES

Sample Analysis: Melatonin

All samples described in Table 1 were analysed for melatonin using the methodologies described in Section 3.

Melatonin was not detected in any sample down to 0.005 µg/ mL.

Sample Screening Analysis: Tryptophan and related compounds

The first 19 samples (LIMS: S18-045307 to S18-045315 and S18-05466 to S18-054474 from Table 1) were screened for all Tryptophan metabolism related compounds in Table 3, using the methodologies described in Section 3.

Only Tryptophan and N-Acetylisatin were detected and were present in all samples. These peak responses are summarised in Table 5. No obvious trends or associations could be made between milking times [night (n=11) or day (n=8)], or between different farms (locations), and the responses of these compounds.

Figures 10 to 13 show the responses of Tryptophan and N-Acetylisatin from a milking time perspective and farm location.

Table 5. Summary of responses by LC-HRMS (peak areas) for Tryptophan and N-Acetylisatin in 19 samples of milk from both farms and over different milking times.

LIMS number	Milking time	Farm	Milk type	N-Acetylisatin response	Tryptophan response
S18-045307	Night	TY Tanglwyst	Pasteurised	43465	8318
S18-045308	Night	TY Tanglwyst	Raw	68887	69171
S18-045309	Night	TY Tanglwyst	Raw	39204	75060
S18-045310	Day	TY Tanglwyst	Raw	62255	63597
S18-045311	Day	TY Tanglwyst	Raw	32038	66147
S18-045312	Day	Haregrove	Raw	34814	58890
S18-045313	Day	Haregrove	Raw	44242	59533
S18-045314	Night	Haregrove	Raw	45623	70631
S18-045315	Night	Haregrove	Raw	51829	76736
S18-054465	Night	TY Tanglwyst	Pasteurised	62859	79516
S18-054466	Night	TY Tanglwyst	Pasteurised	75376	69567
S18-054467	Night	TY Tanglwyst	Raw	36116	76006
S18-054468	Night	TY Tanglwyst	Raw	30247	58880
S18-054469	Day	TY Tanglwyst	Raw	38333	77801
S18-054470	Day	TY Tanglwyst	Raw	44043	73406
S18-054471	Night	Haregrove	Raw	37022	75598
S18-054472	Night	Haregrove	Raw	33786	74900
S18-054473	Day	Haregrove	Raw	45533	73144
S18-054474	Day	Haregrove	Raw	38868	66439

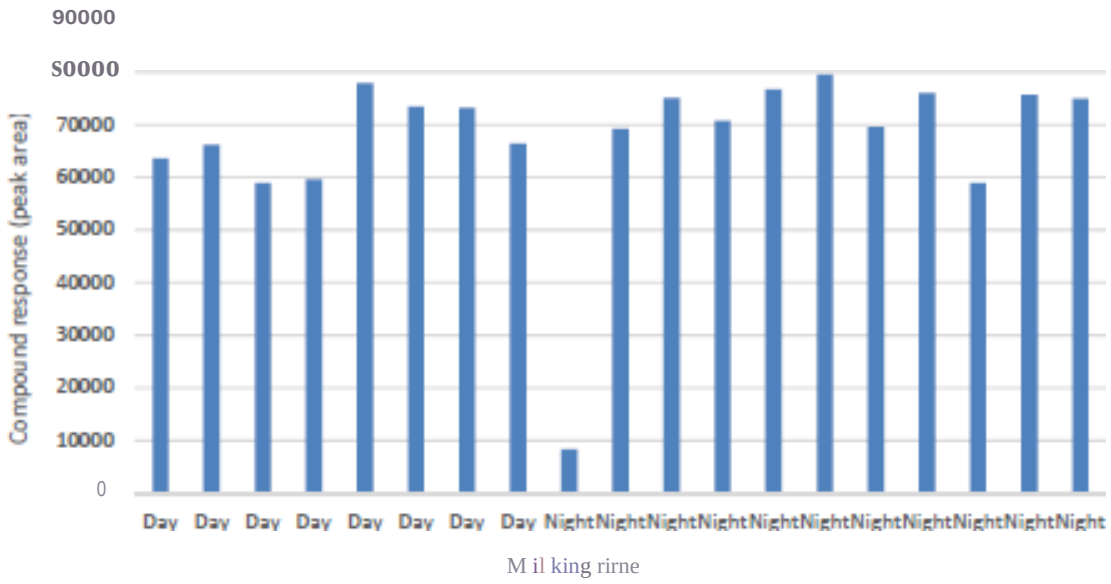


Figure10. Tryptophan response (peakarea) in samples by milking time (night vs day).

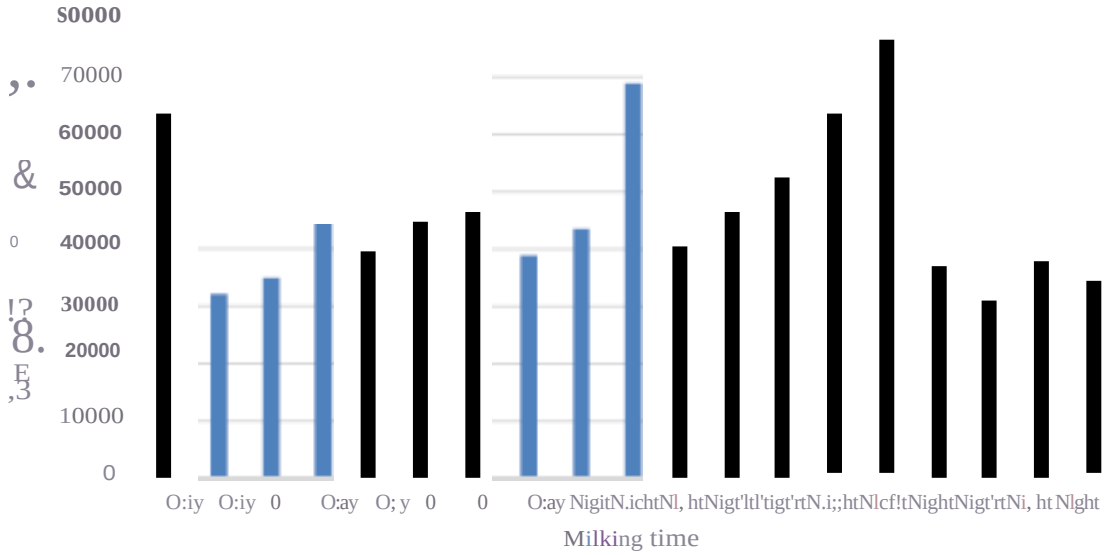


Figure11. N-Acetylisatin respon.se (peakarea) in samples by milking time (night vs day).

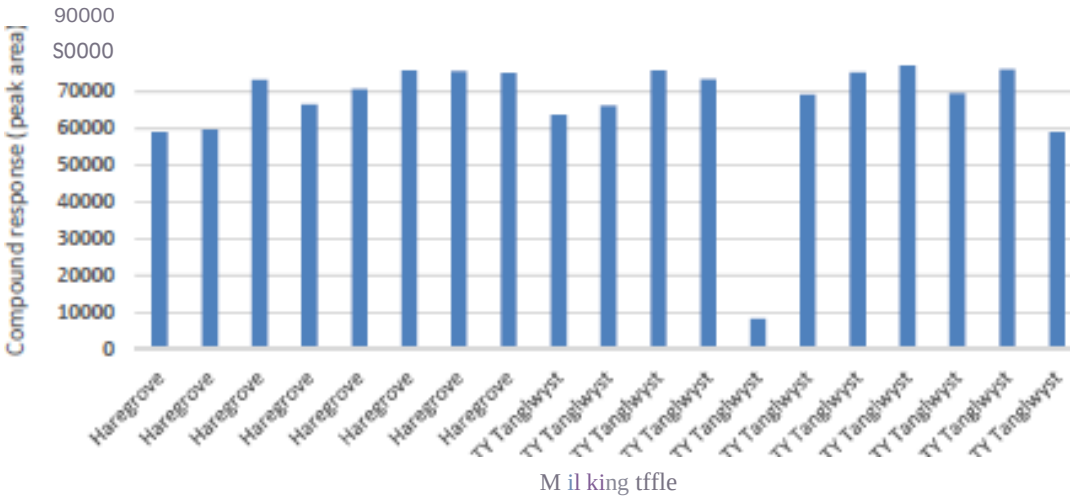


Figure 12. Tryptophan response (peak area) in samples by farm location.

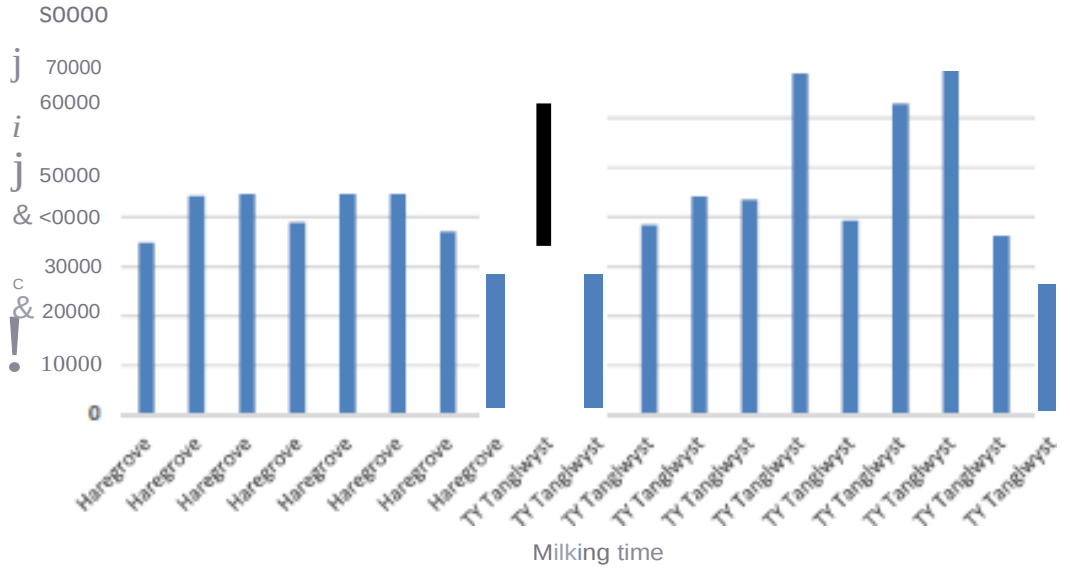


Figure 13. N-Acetylisatin respon.se (peak area) in samples by farm location.

Section 5: CONCLUSIONS

From the 77 samples analysed, no single sample showed any presence of melatonin above 0.005 µg/mL. This indicates there is no elevated level of melatonin, when milking during the night, unless it is at a concentration below these trace limits of detection.

Melatonin is a hormone produced in the tryptophan/serotonin pathway, which regulates diurnal rhythms and influences the reproductive and immune systems. Melatonin synthesis is universally recognised as being regulated by the blue light spectrum (i.e. 446 to 477 nm) in both artificial and sun light. During periods of darkness, it is actively secreted from the pineal gland to induce neural and endocrine effects that regulate circadian rhythms of behavior, physiology, and sleep patterns. However, the human benefit of enhanced sleep patterns from metabolised Melatonin may be better realised from in vivo (ie. within the body) synthesis from the tryptophan amino acid, rather than from consuming enriched melatonin milk. By identifying elevated levels of tryptophan in the milk (which is more than readily available; with milk being the source of the highest levels of tryptophan amongst the common foods) may have the same expected effect perceived from melatonin. As such, this project may have been better placed to scutinise the levels and manipulation of tryptophan within milk.

However, tryptophan was included as a secondary test procedure, but unfortunately, from the first 19 samples analysed for tryptophan and 10 other tryptophan metabolism related compounds only 2 were detected above an estimated concentration of 0.05 µg/ml. These were Tryptophan itself (which as would be expected due to its presence as an amino acid to be present in milk) and the metabolite end product N-Acetylisatin.

Neither of these compounds showed any obvious trend, e.g. an increase or decrease in concentration (based on peak area response), linked to milking time or farm location.

Argument could be made that further examination and a more lengthy programme of testing may identify subtle trends. However, Fera Science believe that the sample testing was sufficiently long and robust to conclude that there is no impact or difference in concentrations within milk produced at different times of the day.