Analysis of effects of Herbabolus on milk quality

M. Groot, M. Alewijn, W. Driessen van Lankveld, A. Lommen, G. Stoopen, D. Venema,
M. Pikkemaat, T. de Rijk

RIKILT
WAGENINGEN UR

Analysis of effects of Herbabolus on milk quality

M. Groot, M. Alewijn, W. Driessen van Lankveld, A. Lommen, G. Stoopen, D. Venema, M. Pikkemaat, T. de Rijk

RIKILT Wageningen UR

Wageningen, March 2015

RIKILT report 2015.007

Groot, M., M. Alewijn, W. Driessen van Lankveld, A. Lommen, G. Stoopen, D. Venema, M. Pikkemaat, T. de Rijk, 2015. *Analysis of effects of Herbabolus on milk quality.* Wageningen, RIKILT Wageningen UR (University & Research centre), RIKILT report 2015.007. 40 pp.; 5 fig.; 1 tab.; 3 ref.

Project number: 1247321201
Project title: Analysis of effects of Herbabolus on milk quality
Project leader: M. Groot

P.O. Box 230, 6700 AA Wageningen, The Netherlands, T +31 (0)317 48 02 56, E info.RIKILT@wur.nl, www.wageningenUR.nl/en/rikilt. RIKILT is part of Wageningen UR (University & Research centre).

RIKILT report 2015.007

Distribution list:
- Biochemproducts B.V.

Contents

Samenvatting

De Herbabolus bestaat uit een mengsel van plantcomponenten op een drager. De bolus is bedoeld om de gezondheid van de koe in de transitieperiode te ondersteunen. De bolus bevat o.a. knoflook (Garlicine), oregano en yucca.

Om de effecten van de bolus op de melkkwaliteit te onderzoek zijn er melkmonsters van koeien met en zonder bolus verzameld en onderzocht op smaak en geur, op antimicrobiële eigenschappen en er is een NMR en LC-MS spectrum gemaakt van melk en van de bolus om te kijken of er signalen van de bolus in de melk waren terug te vinden. Melk van 5 koeien met en 5 koeien zonder bolus is verzameld op dag 0 (voor de bolus), 1, 3, 7 en 14 dagen na de bolusgift. De melk is bij -20°C bewaard tot analyse. Daarnaast zijn twee bolussen geanalyseerd.

Geur en smaak analyse is uitgevoerd met de E-Tong en de vluchtige componenten zijn geanalyseerd met PTR/MS. Gemiddeld over de monsters is er slechts 1 smaakverschil met een waarde die groter is dan 1: richness, voor dagen 1 en 3. Omdat het verschil ook maar net boven de 1 is, en het de ene keer voor het monster met en de andere keer voor het monster zonder bolus is, lijkt dit niet significant, daarvoor is de variatie tussen de 6 monsters te groot. De conclusie is dus dat er geen significante smaakverschillen zijn waargenomen tussen de verschillende melkmonsters met de PTR-MS en de E-tong. Bij de PTR-MS analyse zijn de profielen op elk van de dagen (t=0, 1, 3, 7, 14d) gemiddeld over de koeien in essentie gelijk.

De melkmonsters in dit project zijn getest met de DelvoTest® van DSM. De DelvoTest® wordt door Qlip gebruikt voor de melkcontrole in het kader van de uitbetaling boerderijmelk. Daarnaast is de Multi plaat test uitgevoerd. Alle monsters waren met beide testen negatief.

NMR onderzoek aan de bolus: De bolus is bemonsterd en conform het interne protocol voor regulier monsteronderzoek geanalyseerd t.o.v. de huidige bibliotheek van ca. 1000 spectra van stoffen. Er zijn geen aanwijzingen gevonden voor de aanwezigheid van de stoffen opgenomen in de bibliotheek.

LC-MS (positieve mode ionisatie UHPLC Orbitrap-MS: resolutie 70000) onderzoek aan de bolus: Op basis van element samenstelling zijn annotaties verricht met de complete lijst van de drugbank. Er zijn geen aanwijzingen dat de bolus belangrijke werkzame componenten uit de drugbank bevat.

LC-MS (positieve mode ionisatie UHPLC Orbitrap-MS: resolutie 70000) onderzoek aan de melkmonsters ten behoeve van detectie van bolus-gerelateerde stoffen. Voor dit onderzoek zijn 6 koe-nummers genomen bij tijdstippen 0, 1, 3 en 7 dagen. De 50+ hoogste LC-MS signalen uit punt 2 (bolusprofiel) zijn hiervoor als marker gebruikt. Slechts 2 stoffen (chemische samenstelling resp. $C_{17}H_{34}O_4$ en $C_{19}H_{38}O_4$) bleken boven at random ruis uit te komen in melk. Echter deze 2 kwamen in alle monsters voor (dus ook onbehandelde en onafhankelijk van tijdstip).Er zijn geen signalen gevonden, die direct van de bolus afkomstig zijn

Conclusies

- Er zijn geen significante smaakverschillen waargenomen tussen de melkmonsters van koeien met en zonder bolus, zoals gemeten met de E-tong en PTR-MS.
- De melk monsters van bolus koeien en koeien zonder bolus waren allemaal negatief in de Multi plaat test de DelvoTest® voor antimicrobiële werking.
- Er waren geen verschillen in LC-MS profielen van melk van koeien voor en na de bolus. In de melk van bolus koeien zijn geen signalen gevonden die afkomstig leken te zijn de bolus.

Summary

The Herbabolus is a mix of plant components for cows to improve their health during the transition period. The bolus contains a mixture of herbs including garlic (Garlicin), oregano and yucca.

To investigate the effect of the bolus on milk quality, milk from cows with and without a bolus (control cows) were analysed for odour and taste, for antimicrobial effects and an NMR and LC-MS spectrum was made from the bolus and from the milk to see if similar components were present.

Milk from 5 bolus cows and 5 control cows was sampled at day 0, 1, 3, 7 and 14 and frozen at -20°C until analysis. Moreover two boluses were analysed.

Analysis of taste and odour is performed with the E-tongue and volatile compounds were analysed with PTR/MS. The mean values over all the samples rendered only one taste difference with a value higher than 1 (richness) for day 1 and day 3. Because the difference is just above 1 and it occurs one time in a treated cow and another time in a control cow this does not appear to be significant. Therefore, no significant taste differences were found between milk samples using the E-tongue and PTR-MS.

To investigate possible antimicrobial activity of the milk two tests were performed, the Multi (7) plate test and the tube test (DelvoTest®). None of the samples were tested as suspect in either of the tests.

To analyse the composition of the bolus an Nuclear Magnetic Resonance Spectroscopy (NMR) profile conform regular sample analysis and an LC-MS profile was made in threefold. The bolus was sampled conform the internal protocol for regular sample analysis and analysed for known spectra of 1000 compounds currently present within the reference library. We did not find any known spectra matching these compounds.

LC-MS (positive mode ionisation UHPLC Orbitrap-MS: resolution 70000) investigation of milk samples for retrieving bolus related compounds in the milk. For this 6 cow numbers (2 control and 4 bolus) have been analysed at day 0, 1, 3 and 7. The 50+ highest LC-MS signals from the bolus profile were used as a marker for this. Only two compounds (chemical composition resp. $C_{17}H_{34}O_4$ en $C_{19}H_{38}O_4$) appeared to be present above background noise in the milk. But these compounds were present in all milk samples and unrelated to treatment or time of sampling. We concluded that in the milk of treated cows no signals were found that appeared to come directly from the bolus.

Conclusions

- No significant taste differences were found between milk samples using the E-tongue and PTR-MS.
- The milk samples of bolus and non-bolus cows were all negative in the Multiplate test and DelvoTest® for antimicrobial activity.
- There were no differences in milk profiles from cows before or after the bolus. In the milk of treated cows no signals were found that appeared to come directly from the bolus.

1 Introduction

An inventory of the use of antibiotics in dairy cattle showed that 60 % of the total use was preventive use in the dry period. Since January 13th 2014 the preventative use of antibiotics is no longer allowed in the Netherlands. The aim of drying off cows with antibiotics was 1) curing of new infections and 2) prevention of new infections.

Reduction of the use of antibiotics will require better management from the farmers. Products that reduce the risk of new infections will be highly appreciated.

The company Biochemproducts B.V. sells a Herbabolus as a means to reduce the somatic cell count in dairy cows and to protect the animals in the dry period. The bolus is given to the animal 7 days before the dry period.

This bolus contains a mixture of herbs including garlic (Garlicine), oregano and yucca. Garlic and oregano have antimicrobial effects (Wichtl, 2002), yucca has antimicrobial and anti-inflammatory effects (Cheeke, P.R, 2001). These effects may be beneficial for the cow but antimicrobial effects may influence the suitability of the milk for cheese making. The herbs used have a strong odour which might influence the odour and quality of the milk. Moreover residues of the product might be excreted via the milk.

To investigate the effect of the bolus on milk quality, milk from cows with and without a bolus (control cows) were analysed for odour and taste, for antimicrobial effects and an NMR and LC-MS spectrum was made from the bolus and from the milk to see if similar components were present.

2 Material and Methods

2.1 Animal experiment

To investigate the differences between cows supplied with a bolus and cows without a bolus two groups of 5 animals (5 with bolus and 5 without bolus) were followed in time.

During 14 days animals were sampled at day 0, 1, 3, 7 and 14 and also a sample of the milk tank was taken, see Table 1. The data of the cows are listed in Annex 1.

Table 1
Sampling scheme milk.

Sampling day	0	1	3	7	14
Bolus	X	X	X	X	X
Control	X	X	X	X	X
Tank milk	X	x	X	X	X

2.2 Flavour compound analysis

Analysis of taste and odour is performed with the E-tongue. The electronic tongue is an instrument that measures and compares tastes. Chemical compounds responsible for taste are detected by human taste receptors, and the seven sensors of electronic instruments detect the same dissolved organic and inorganic compounds. Like human receptors, each sensor has a spectrum of reactions different from the other. The information given by each sensor is complementary and the combination of all sensors' results generates a unique fingerprint. The detection thresholds of sensors are similar to those of human receptors.

Figure 1 *E-Tongue.*

The tastes that can be recognised by the E-Tongue are: sourness, bitterness, saltiness, umami taste enhancer, astringency, and several aftertastes thereof (astringency (aftertaste a) and bitterness (aftertaste b) and umami (richness).

For the E-tongue all samples are measured in undependable replicates for 4 times. The first cycle is a conditioning step for the sensors. The mean potentiometric signals of the other cycles are recalculated into taste values using the specific E-Tongue software. The absolute values are not relevant, we focus on possible differences between the samples. Differences in taste values > 1 ale likely to be discerned by humans, whereas smaller differences generally do not influence the observed taste.

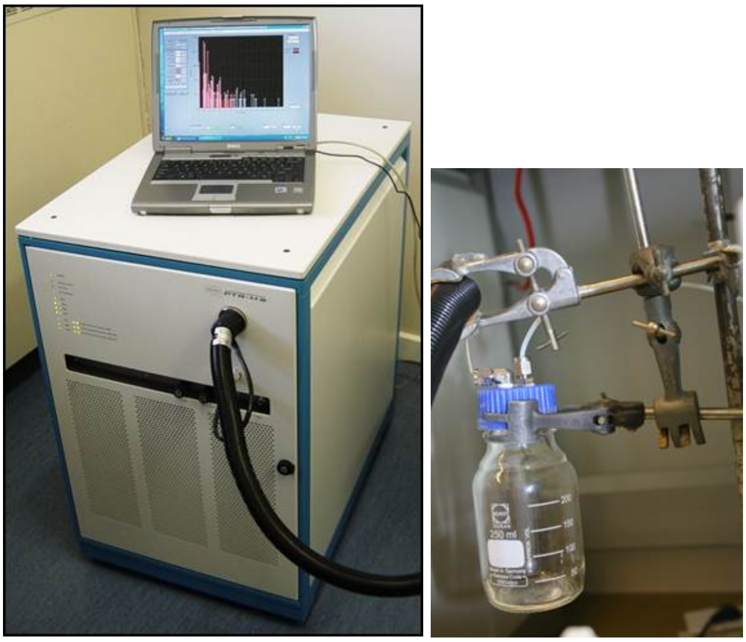


Figure 2 *PTR/MS and left the headspace sampling (thin layer of milk in a 250 ml bottle).*

PTR/MS: Proton Transfer Reaction / Mass Spectrometry is the benchmark method for simultaneous real-time monitoring of volatile (organic) compounds. The PTR measures all volatile organic compounds present in the headspace. Before transferring the milk to the machine it is kept for 30 minutes at room temperature in a closed bottle in shaking bath. All measurements are performed in duplicate with the PTR full scan mode of m/z 20-160. The PTR/MS detects volatile compounds (possible aroma compound) and is largely complementary to the E-tongue, which measures non-volatile taste compounds.

2.3 Antimicrobial analysis

To investigate eventual antimicrobial activity of the milk two tests are performed:
- Multi (7) plate test
- Tube test

Both tests are based on bacterial growth inhibition. The multi plate test consists of 7 plates that are specific for a group of antibiotics. This test will detect almost all antibiotics at their EU Maximum Residue Limit (MRL). The method (SOP A0717) is used by RIKILT Wageningen UR to test milk samples on behalf of the NVWA (Dutch Food Safety Authority)within the framework of the National Monitoring Program. The method used is an improved version of the method published by Nouws (1999).

Milk samples are applied into holes in each of the 7 plates and after overnight incubation the samples are evaluated. The presence of an inhibition zone indicates antimicrobial activity of the sample (Figure 3).

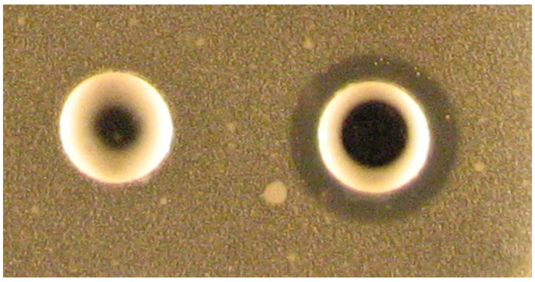


Figure 3 *Example of a negative sample (left) and an inhibition zone due to antimicrobial activity (right).*

Next to the Multi plate test we performed a tube test, in this case the DelvoTest® from DSM. This is an ampule which contains a test bacterium, Bacillus stearothermophilus, and a growth medium with a pH indicator. The milk sample is applied to the growth medium and incubated for 3-3,5 hours at 64°C. This induces the bacterium to grow and produce acid which will induce a colour change from purple to yellow. Samples with antimicrobial activity will inhibit bacterial growth and subsequently will not show a colour change. Delvotest is very sensitive for beta-lactam antibiotics, but many other groups of antibiotics will only be found positive at levels > MRL. The DelvoTest® is used by Qlip for milk control in the framework of payment for farm milk.

Figure 4 *DelvoTest®, negative sample on the left (yellow) and positive sample right (purple).*

2.4 Chemical analysis

2.4.1 Bolus composition

To analyse the composition of the bolus an Nuclear Magnetic Resonance Spectroscopy (NMR) profile conform regular sample analysis and an LC-MS profile was made in threefold.

Over the past fifty years NMR, has become the preeminent technique for determining the structure of organic compounds. NMR generates a complete analysis of all molecules present in the sample and the entire spectrum is normally interpreted.

LC-MS: Liquid chromatography–mass spectrometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS). LC-MS is a powerful technique that has very high sensitivity and selectivity and so is useful in many applications. Its application is oriented towards the separation, general detection and potential identification of chemicals of particular masses in the presence of other chemicals (i.e., in complex mixtures), e.g., natural products from natural-products extracts, and pure substances from mixtures of chemical intermediates. Preparative LC-MS systems can be used for rapid mass-directed purification of specific substances from such mixtures that are important in basic research, and pharmaceutical, agrochemical, food, and other industries.

2.4.2 Changes in milk composition, residues bolus

To analyse the composition of the bolus an Nuclear Magnetic Resonance Spectroscopy (NMR) profile conform regular sample analysis and an LC-MS profile was made in threefold. Milk is analysed in a similar way and then the profile of the milk is checked for the most prominent signals from the bolus at day 0, 1 and 3 (3 times). Bolus milk is compared with control milk.

3 Results

3.1 Aromatic analysis

3.1.1 E-tongue

The mean values over all the samples rendered only one taste difference with a value higher than 1 (richness) for day 1 and day 3. Because the difference is just above 1 and it occurs one time in a treated cow and another time in a control cow this does not appear to be significant. We can conclude that the bolus does not affect the taste of the milk. The results are listed in Annex 2.

Some individual samples can be distinguished from others based on the difference in taste value> 1, but there were no trends for different cows, days or treatments.

3.1.2 PTR-MS

We tried to find differences between samples of cows with and without bolus between all samples and between samples of day 0 and day 1. We used statistical techniques, principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to look for differences between the different milk samples, and to find possible (multivariate) differences between bolus and non-bolus milk, respectively. It was assumed that with bolus some masses (molecular weights) would increase in intensity or possibly decrease due to absorption. None of these were found. The means of the profiles of the cows were the same on each of the sampling days (0, 1, 3, 7 and 14). For the plots we used fourth power root scaling.

The differences that are observed in the plots do not have a trend relation in time, and are probably not related to treatment. There are differences between the different sampling days and between different cows. For data see Annex 3.

3.2 Antimicrobial analysis

All samples were negative with the Multi-plate test as well as with the DelvoTest®. For individual results see Annex 4.

3.3 Chemical analysis

3.3.1 Analysis bolus

NMR analysis bolus. The bolus is sampled conform the internal protocol for regular sample analysis and analysed for known spectra (1000) within the library. We did not find any known spectra.

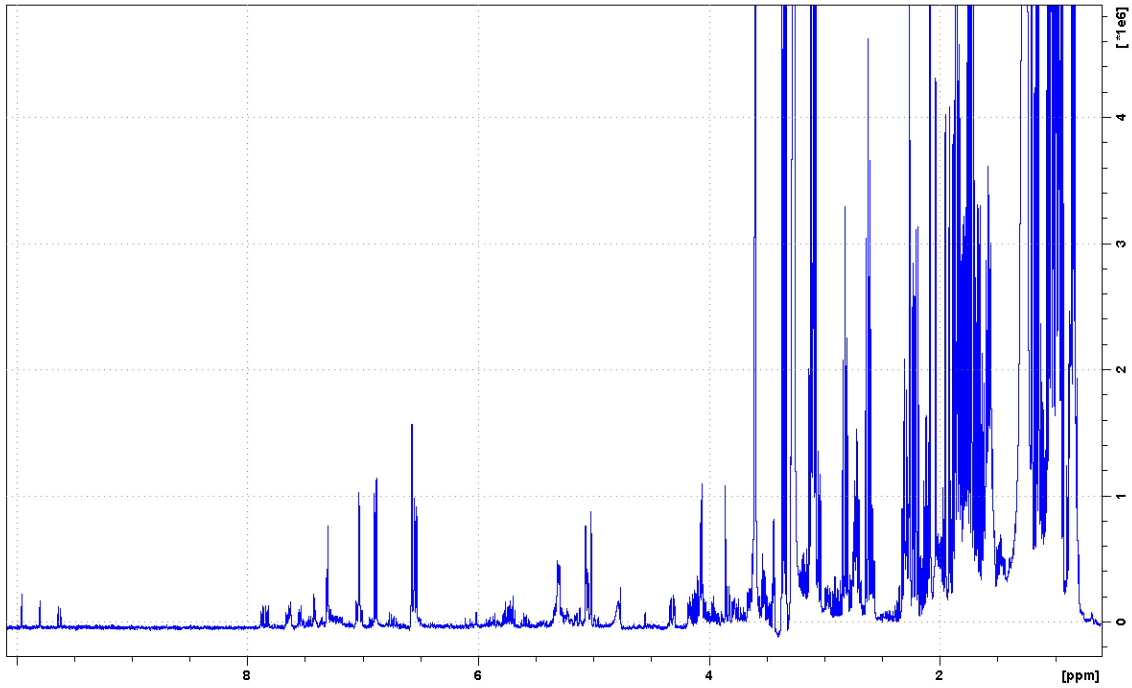


Figure 5 *NMR spectrum of the herbabolus.*

LC-MS (positive mode ionisation UHPLC Orbitrap-MS: resolution 70000) analysis of the bolus. The bolus is samples 3 times and processes for analysis. Because there was no placebo bolus we made a sample of control chemicals. This is needed to filter background signals from the chemical profile. The bolus and the control chemicals are processed and compared using the metAlign software. The resulting pure bolus profile is broad analysed by element composition of the whole profile. Based on the element composition annotations were performed with the complete list of the drug bank (http://www.drugbank.ca/). It was concluded that the bolus did not contain active components from the drugs bank. For the LC-MS spectra of the cows on different days see Annex 5.

3.3.2 Analysis milk for residues

LC-MS (positive mode ionisation UHPLC Orbitrap-MS: resolution 70000) investigation of milk samples for retrieving bolus related compounds in the milk. For this 6 cow numbers (3 control and 3 bolus) have been analysed at day 0, 1, 3 and 7. The 50+ highest LC-MS signals from the bolus profile were used as a marker for this. Only two compounds (chemical composition resp. $C_{17}H_{34}O_4$ and $C_{19}H_{38}O_4$) appeared to be present above background noise in the milk. But these compounds were present in all milk samples and unrelated to treatment or time of sampling. We concluded that in the milk of treated cows no signals were found that appeared to come directly from the bolus. It is however possible that the compounds from the bolus have been metabolized and not recognized in the milk.

Conclusions

- No significant taste differences were found between milk samples using the E-tongue and PTR-MS.
- The milk samples of bolus and non-bolus cows were all negative in the Multiplate test and DelvoTest® for antimicrobial activity.
- There were no differences in milk profiles from cows before or after the bolus. In the milk of treated cows no signals were found that appeared to come directly from the bolus.

References

Cheeke, P.R. 2001. Actual and potential applications of Yucca schidigera and Quillaja saponaria saponins in human and animal nutrition. http://livestocklibrary.com.au/handle/1234/19910.

Nouws, J., Van Egmond, H., Smulders, I., Loeffen, G., Schouten, J., Stegeman, H. 1999. Document A microbiological assay system for assessment of raw milk exceeding EU maximum residue levels. International Dairy Journal 9, 85-90.

Wichtl, M. (ed.) 2002. Herbal drugs and phytopharmaceuticals. A handbook for practice on a scientific basis. CRC Press, Boca Raton, London, New York, Washington DC.

Acknowledgement

This work was funded by Biochemproducts B.V.

Annex 1 Milk samples and cow data

Samples Biochemproducts milk

Date	Day nr.	sample code	Cow nr.	kg milk	parity	Lactation stage	Somatic Cell count	fat %	protein%
1-12-2014	0	V1	2376	35,05	1	91	580	3,76	3,2
	0	V2	2150	34,63	5	75	1099	4,43	4,05
	0	V3	2317	28,77	2	96	418	4,19	3,63
	0	V4	2173	25,05	4	228	483	5,08	3,86
	0	V5	2106	16,2	5	182	296	4,53	3,49
	0	V6	2320	34,03	2	47	764	4,55	3,46
	0	V7	2203	28,94	4	111	75	3,46	3,34
	0	V8	2140	37,12	5	125	78	5,13	3,81
	0	V9	2365	28,2	1	137	83	4,63	3,78
	0	V10	2054	14,26	6	362	212	6,35	4,73
		V11	tank milk						
2-12-2014	1	V12	2376						
	1	V13	2150						
	1	V14	2317						
	1	V15	2173						
	1	V16	2106						
	1	V17	2320						
	1	V18	2203						
	1	V19	2140						
	1	V20	2365						
	1	V21	2054						
		V22	tank milk						
4-12-2014	3	V23	2376						
	3	V24	2150						
	3	V25	2317						
	3	V26	2173						
	3	V27	2106						
	3	V28	2320						
	3	V29	2203						
	3	V30	2140						
	3	V31	2365						
	3	V32	2054						
		V33	tank milk						
8-12-2014	7	V34	2376						
	7	V35	2150						
	7	V36	2317						
	7	V37	2173						
	7	V38	2106						
	7	V39	2320						
	7	V40	2203						
	7	V41	2140						
	7	V42	2365						
	7	V43	2054						
		V44	tank milk						
15-12-2014	14	V45	2376						
	14	V46	2150						
	14	V47	2317						
	14	V48	2173						
	14	V49	2106						
	14	V50	2320						
	14	V51	2203						
	14	V52	2140						
	14	V53	2365						
	14	V54	2054						
		V55	tank milk						

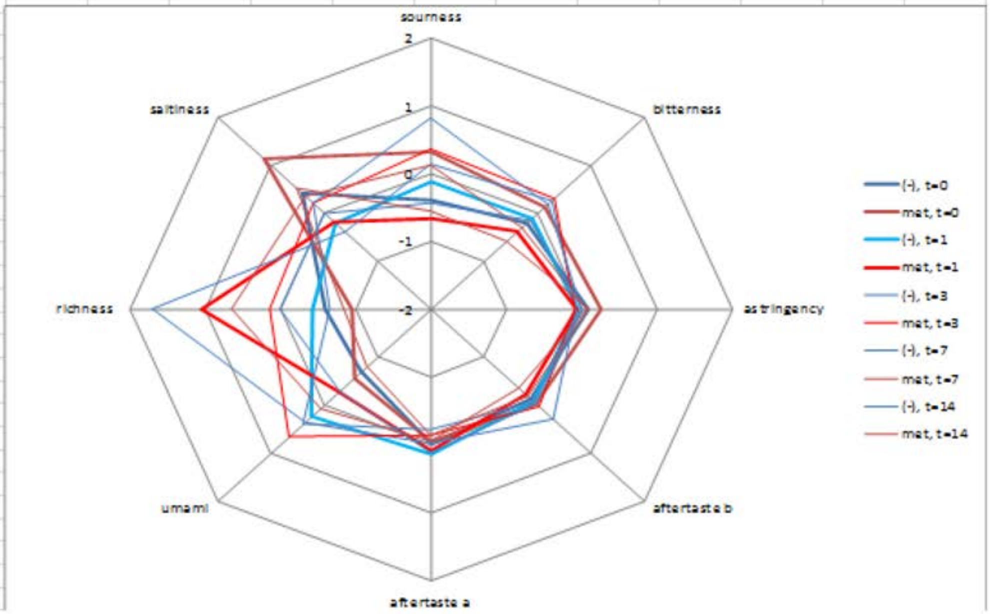
Annex 2 Results E-tongue

E-tongue

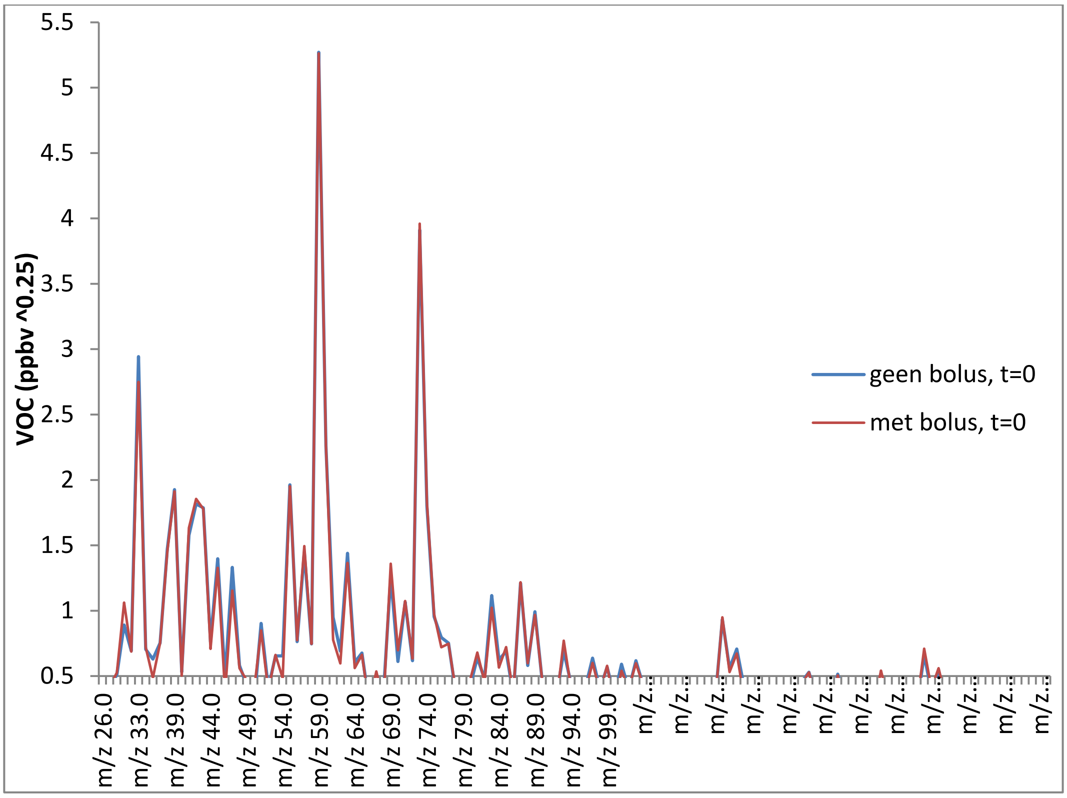
			Taste values							
sample	dag nr	bolus 1 zo	sourness	bitterness	astringen	aftertaste	aftertaste	umami	richness	saltiness
2054	0	1	-19.39	3.57	-7.30	1.72	1.64	12.12	14.16	15.62
2140	0	1	-21.19	2.73	-7.70	1.48	1.28	12.40	11.96	15.28
2173	0	1	-21.72	2.13	-8.01	1.21	1.35	12.51	11.58	16.02
2203	0	1	-21.52	1.97	-7.71	1.09	1.35	12.49	11.75	16.04
2376	0	1	-21.45	1.31	-8.44	0.96	1.55	12.42	10.82	16.85
tank milk	0	1	-20.26	2.23	-7.93	1.43	0.99	12.36	13.42	14.76
2106	0	2	-21.33	1.82	-7.95	1.00	1.09	12.32	12.03	16.23
2150	0	2	-22.19	2.15	-8.41	1.08	1.53	12.67	11.82	17.24
2317	0	2	-22.00	2.55	-8.17	1.29	1.32	12.64	11.42	16.39
2320	0	2	-21.70	2.44	-7.87	1.21	1.27	12.60	11.44	16.34
2365	0	2	-20.92	4.33	-7.67	2.45	1.79	12.35	12.97	16.26
2054	1	1	-21.87	1.62	-7.80	1.26	1.39	14.12	10.69	15.61
2140	1	1	-21.93	2.33	-7.61	1.55	1.37	14.10	10.56	14.90
2173	1	1	-19.84	2.52	-7.36	1.10	1.01	12.22	13.71	14.67
2203	1	1	-20.71	2.81	-7.81	1.12	1.24	12.71	14.87	15.52
2376	1	1	-20.66	3.58	-7.79	1.62	1.19	12.59	13.51	15.14
tank milk	1	1	-22.11	1.68	-7.96	1.46	1.01	14.20	11.42	15.08
2106	1	2	-20.87	0.67	-8.06	0.46	1.02	12.71	15.10	16.00
2150	1	2	-20.56	2.54	-7.80	1.22	1.14	12.51	13.41	14.80
2317	1	2	-21.09	1.26	-8.00	0.56	0.91	12.76	13.57	14.89
2320	1	2	-20.44	3.11	-7.72	1.35	1.25	12.57	15.02	14.97
2365	1	2	-20.25	3.17	-6.92	2.50	2.00	13.52	12.58	15.22
2054	3	1	-21.06	3.39	-7.75	2.41	1.66	13.11	17.60	15.09
2140	3	1	-21.73	2.08	-8.00	1.38	1.19	13.38	15.87	15.10
2173	3	1	-21.82	2.80	-7.56	1.54	1.36	14.04	11.58	15.09
2203	3	1	-20.98	2.82	-7.62	1.68	1.40	12.96	14.54	14.73
2376	3	1	-21.73	3.10	-7.43	1.61	1.36	13.97	11.50	15.05
tank milk	3	1	-21.36	2.54	-7.83	1.78	1.23	13.20	16.36	14.67
2106	3	2	-21.95	2.60	-7.67	1.09	1.38	14.15	11.21	15.87
2150	3	2	-22.44	2.51	-8.28	0.98	1.27	14.30	9.83	15.95
2317	3	2	-21.94	1.31	-7.80	0.80	1.21	14.07	11.85	15.17
2320	3	2	-20.76	3.94	-7.07	2.51	2.11	12.90	17.29	15.22
2365	3	2	-21.24	3.82	-7.58	1.91	1.48	13.29	14.93	15.59
2054	7	1	-22.52	2.36	-7.93	1.21	1.83	13.71	13.16	16.18
2140	7	1	-22.30	4.08	-7.62	1.59	1.72	13.66	12.18	16.05
2173	7	1	-22.44	2.50	-7.79	1.09	1.62	13.41	10.04	16.17
2203	7	1	-22.41	2.21	-7.90	1.10	1.36	13.65	12.48	15.53
2376	7	1	-20.94	3.15	-7.40	1.81	1.40	12.91	14.88	14.20
tank milk	7	1	-22.20	2.04	-7.90	1.44	1.52	13.54	10.64	15.21
2106	7	2	-21.70	2.25	-7.91	0.98	1.54	13.22	10.26	16.31
2150	7	2	-21.70	1.61	-8.15	1.34	1.27	13.23	16.16	15.44
2317	7	2	-20.94	1.39	-7.65	0.77	1.18	12.93	14.86	15.18
2320	7	2	-20.85	2.73	-7.57	0.72	1.48	12.94	13.27	15.58
2365	7	2	-22.00	3.20	-7.84	1.63	1.55	13.47	13.13	15.99
2054	14	1	-20.08	1.98	-7.71	1.60	1.15	12.72	16.34	15.02
2140	14	1	-21.16	2.05	-8.13	1.60	1.09	13.07	14.77	15.63
2173	14	1	-20.81	3.82	-7.28	1.37	1.36	12.00	9.62	14.93
2203	14	1	-20.76	1.91	-7.77	1.38	1.30	12.78	13.41	16.11
2376	14	1	-21.50	2.56	-7.41	0.71	1.59	12.93	8.17	14.52
tank milk	14	1	-21.01	1.99	-8.37	1.82	0.95	13.27	14.96	15.91
2106	14	2	-20.64	3.03	-7.26	1.09	1.28	12.00	10.52	15.48
2150	14	2	-21.20	2.30	-8.17	1.08	1.91	11.92	10.34	16.53
2317	14	2	-21.00	0.93	-7.82	0.42	1.03	12.01	8.50	15.09
2320	14	2	-20.27	1.14	-7.66	2.11	2.12	12.42	15.47	16.39
2365	14	2	-20.71	2.30	-8.36	1.55	1.12	13.07	15.35	15.87

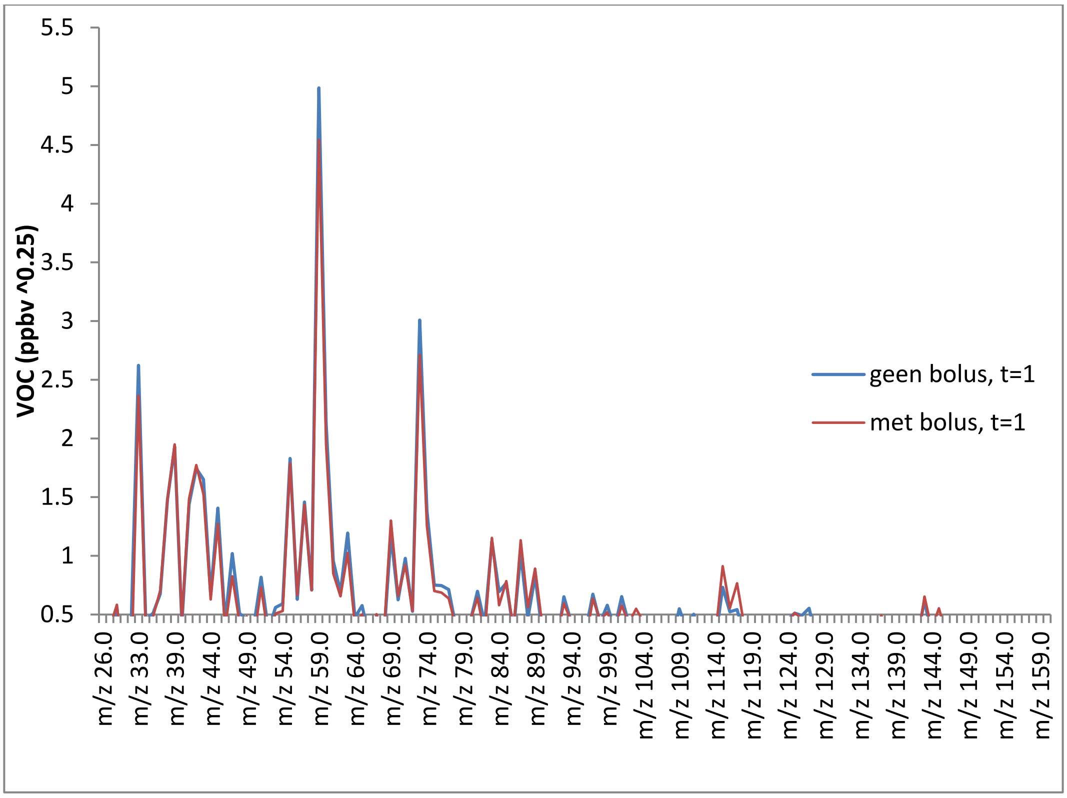
		Averaged taste values							
day nr	bolus 1 zo	sourness	bitterness	astringen	aftertaste	aftertaste	umami	richness	saltiness
0	1	-20.92	2.32	-7.85	1.32	1.36	12.38	12.28	15.76
0	2	-21.63	2.66	-8.01	1.41	1.40	12.52	11.94	16.49
1	1	-21.19	2.42	-7.72	1.35	1.20	13.32	12.46	15.15
1	2	-20.65	2.15	-7.70	1.22	1.27	12.81	13.94	15.17
3	1	-21.45	2.79	-7.69	1.73	1.37	13.44	14.57	14.96
3	2	-21.67	2.84	-7.68	1.46	1.49	13.74	13.02	15.56
7	1	-22.14	2.72	-7.76	1.38	1.58	13.48	12.23	15.56
7	2	-21.44	2.23	-7.83	1.09	1.41	13.16	13.53	15.70
14	1	-20.89	2.38	-7.78	1.41	1.24	12.79	12.88	15.36
14	2	-20.77	1.94	-7.85	1.25	1.50	12.28	12.04	15.88
averaged without bolus		*-21.32*	*2.53*	*-7.76*	*1.44*	*1.35*	*13.08*	*12.88*	*15.36*

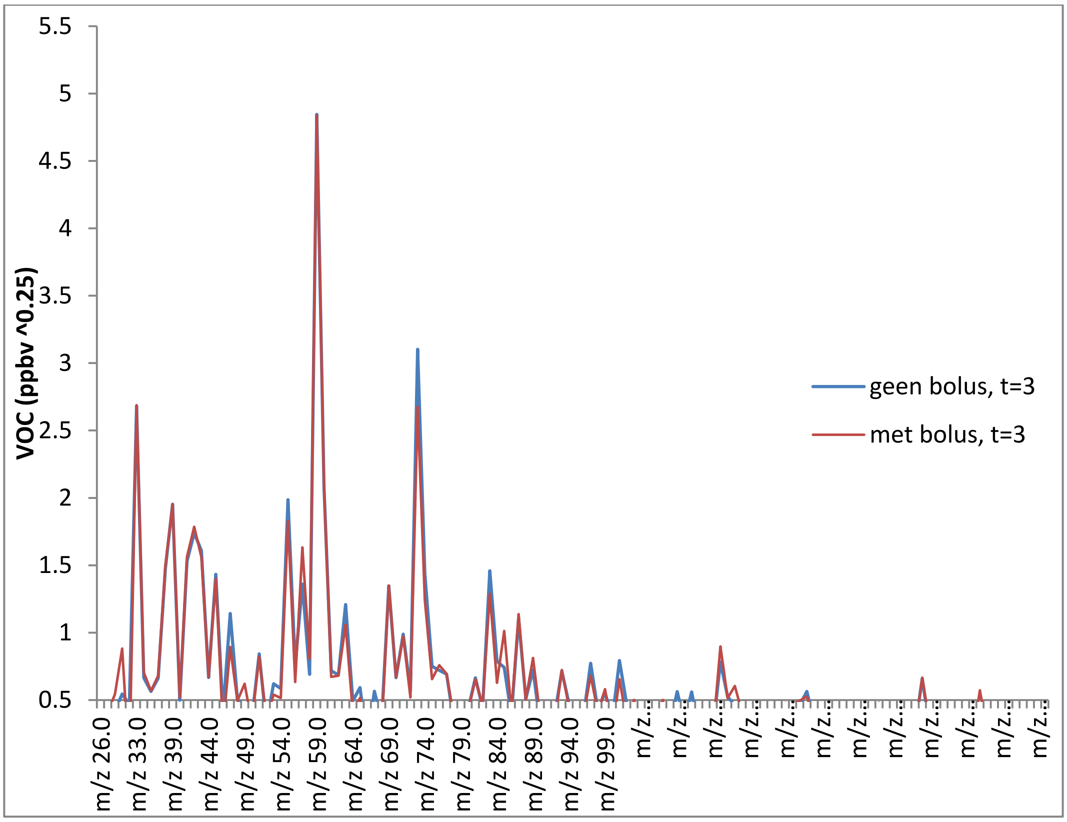
		Taste values relative to averaged blank milk sample							
		Values >1 or <-1 indicate discernible differences							
day nr	bolus 1 zo	sourness	bitterness	astringen	aftertaste	aftertaste	umami	richness	saltiness
0	1	-0.40	-0.20	0.09	-0.12	-0.01	-0.70	-0.60	0.40
0	2	0.31	0.13	0.25	-0.03	-0.05	-0.57	-0.95	1.14
1	1	-0.13	-0.11	-0.04	-0.09	0.15	0.24	-0.43	-0.21
1	2	-0.67	-0.38	-0.06	-0.22	0.08	-0.27	1.05	-0.18
3	1	0.13	0.26	-0.07	0.30	-0.02	0.36	1.69	-0.40
3	2	0.35	0.31	-0.08	0.02	-0.14	0.66	0.14	0.21
7	1	0.82	0.19	0.00	-0.06	-0.23	0.39	-0.66	0.20
7	2	0.12	-0.29	0.07	-0.35	-0.06	0.07	0.65	0.34
14	1	-0.43	-0.15	0.02	-0.03	0.11	-0.29	0.00	0.00
14	2	-0.55	-0.59	0.09	-0.19	-0.15	-0.80	-0.84	0.52



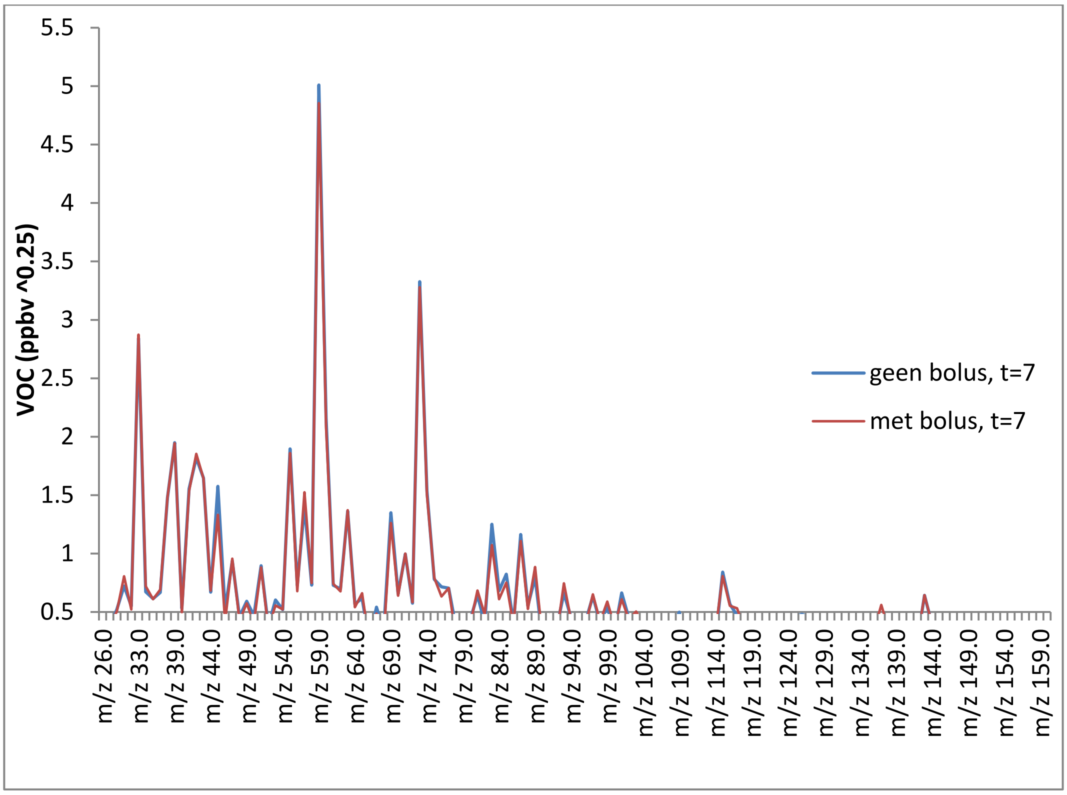
Annex 3 PTR-MS spectra of the milk with and without bolus on different sampling days



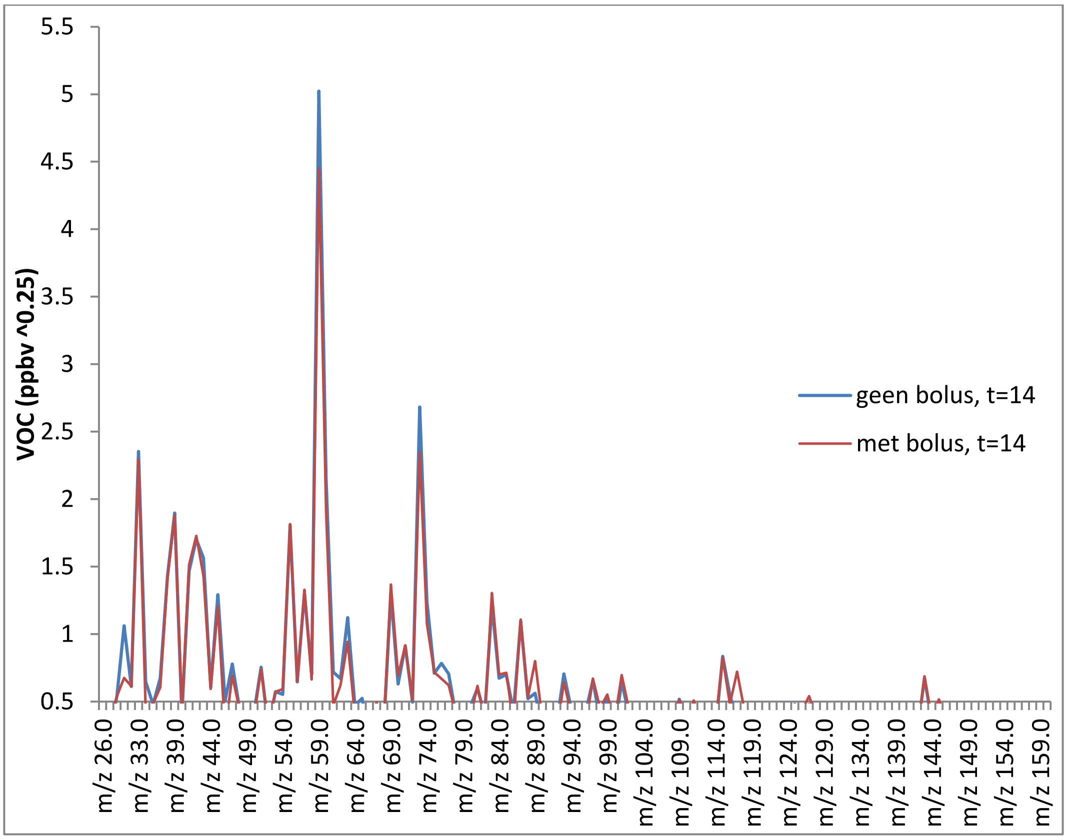




VOC (ppbv ^0.25)
geen bolus, t=3
met bolus, t=3



VOC (ppbv ^0.25)
geen bolus, t=7
met bolus, t=7



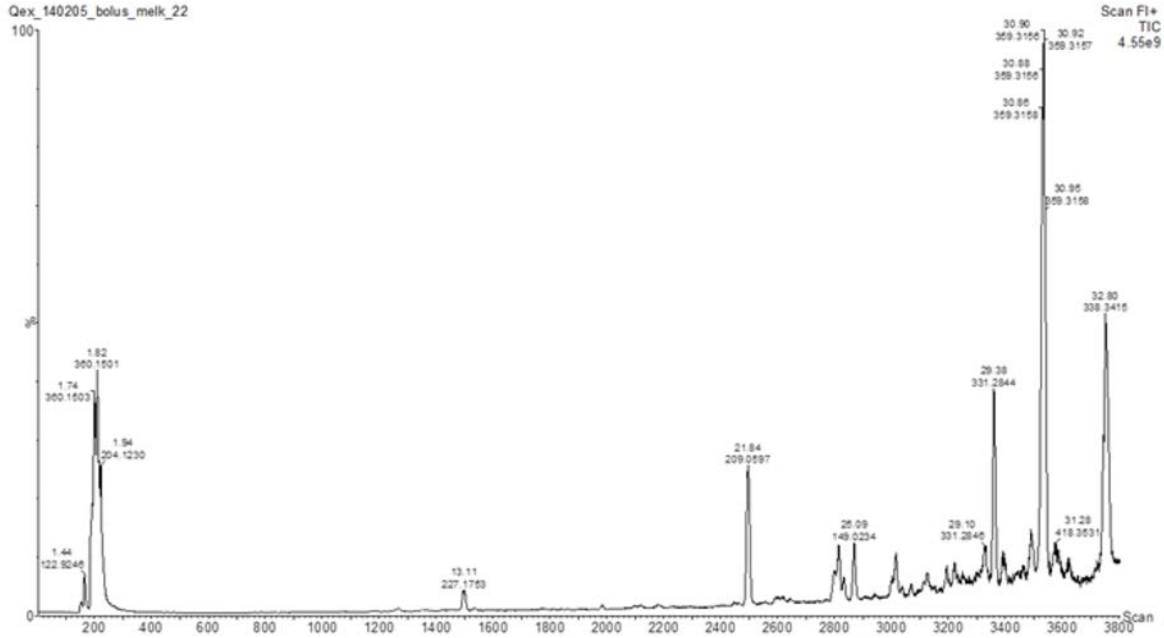
VOC (ppbv ^0.25)
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
geen bolus, t=14
met bolus, t=14
m/z 26.0
m/z 33.0
m/z 39.0
m/z 44.0
m/z 49.0
m/z 54.0
m/z 59.0
m/z 64.0
m/z 69.0
m/z 74.0
m/z 79.0
m/z 84.0
m/z 89.0
m/z 94.0
m/z 99.0
m/z 104.0
m/z 109.0
m/z 114.0
m/z 119.0
m/z 124.0
m/z 129.0
m/z 134.0
m/z 139.0
m/z 144.0
m/z 149.0
m/z 154.0
m/z 159.0

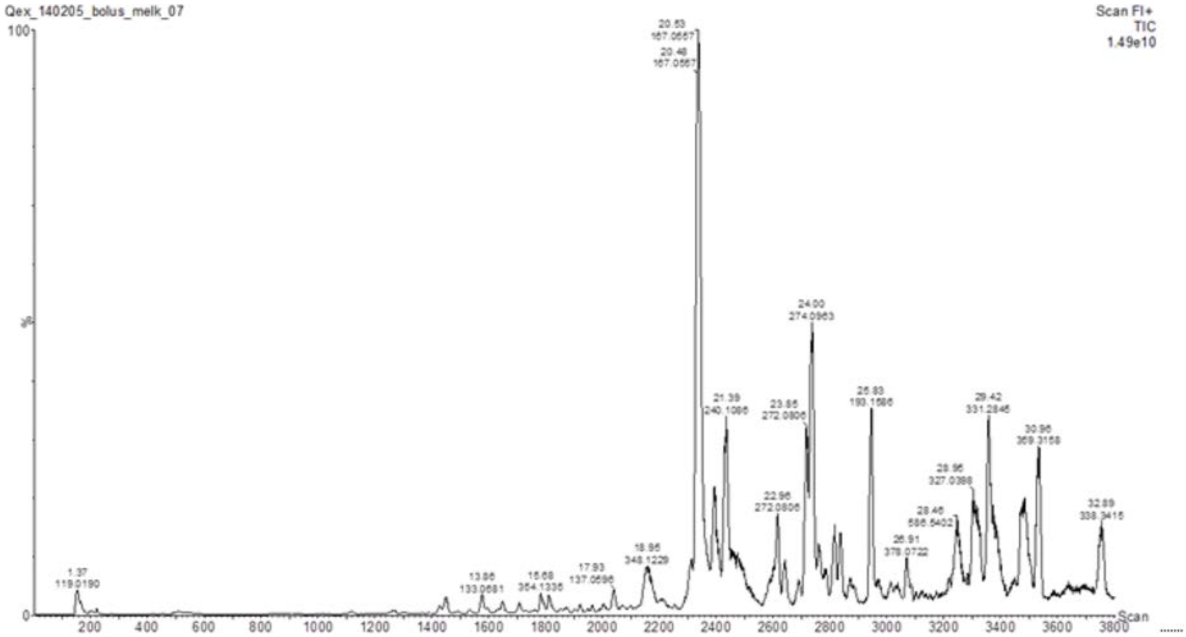
Annex 4 Milk analysis for antimicrobial action

Date	Daynr.	Sample code	Cow nr.	RIKILT nr	BACTERIOLOGY Analysis antimicrobial activity	
					SOP A718 sectie A (Tube test)	SOP A 0717 (plate test)
1-12-2014	0	B1	2376	359027	Non-suspect	Non-suspect
	0	B2	2150	359028	Non-suspect	Non-suspect
	0	B3	2317	359029	Non-suspect	Non-suspect
	0	B4	2173	359030	Non-suspect	Non-suspect
	0	B5	2106	359031	Non-suspect	Non-suspect
	0	B6	2320	359032	Non-suspect	Non-suspect
	0	B7	2203	359033	Non-suspect	Non-suspect
	0	B8	2140	359034	Non-suspect	Non-suspect
	0	B9	2365	359035	Non-suspect	Non-suspect
	0	B10	2054	359036	Non-suspect	Non-suspect
		B11	tank milk	359037	Non-suspect	Non-suspect
2-dec	1	B12	2376	359038	Non-suspect	Non-suspect
	1	B13	2150	359039	Non-suspect	Non-suspect
	1	B14	2317	359040	Non-suspect	Non-suspect
	1	B15	2173	359041	Non-suspect	Non-suspect
	1	B16	2106	359042	Non-suspect	Non-suspect
	1	B17	2320	359043	Non-suspect	Non-suspect
	1	B18	2203	359044	Non-suspect	Non-suspect
	1	B19	2140	359045	Non-suspect	Non-suspect
	1	B20	2365	359046	Non-suspect	Non-suspect
	1	B21	2054	359047	Non-suspect	Non-suspect
		B22	tank milk	359048	Non-suspect	Non-suspect
	3	B23	2376	359816	Non-suspect	Non-suspect
	3	B24	2150	359817	Non-suspect	Non-suspect
	3	B25	2317	359818	Non-suspect	Non-suspect
	3	B26	2173	359819	Non-suspect	Non-suspect
	3	B27	2106	359820	Non-suspect	Non-suspect
	3	B28	2320	359821	Non-suspect	Non-suspect
	3	B29	2203	359822	Non-suspect	Non-suspect
	3	B30	2140	359823	Non-suspect	Non-suspect
	3	B31	2365	359824	Non-suspect	Non-suspect
	3	B32	2054	359825	Non-suspect	Non-suspect
		B33	tank milk	359826	Non-suspect	Non-suspect
	7	B34	2376	359827	Non-suspect	Non-suspect
	7	B35	2150	359828	Non-suspect	Non-suspect
	7	B36	2317	359829	Non-suspect	Non-suspect
	7	B37	2173	359830	Non-suspect	Non-suspect
	7	B38	2106	359831	Non-suspect	Non-suspect
	7	B39	2320	359832	Non-suspect	Non-suspect
	7	B40	2203	359833	Non-suspect	Non-suspect
	7	B41	2140	359834	Non-suspect	Non-suspect
	7	B42	2365	359835	Non-suspect	Non-suspect
	7	B43	2054	359836	Non-suspect	Non-suspect
		B44	tank milk	359837	Non-suspect	Non-suspect
	14	B45	2376	359838	Non-suspect	Non-suspect
	14	B46	2150	359839	Non-suspect	Non-suspect
	14	B47	2317	359840	Non-suspect	Non-suspect
	14	B48	2173	359841	Non-suspect	Non-suspect
	14	B49	2106	359842	Non-suspect	Non-suspect
	14	B50	2320	359843	Non-suspect	Non-suspect
	14	B51	2203	359844	Non-suspect	Non-suspect
	14	B52	2140	359845	Non-suspect	Non-suspect
	14	B53	2365	359846	Non-suspect	Non-suspect
	14	B54	2054	359847	Non-suspect	Non-suspect
		B55	tank milk	359848	Non-suspect	Non-suspect

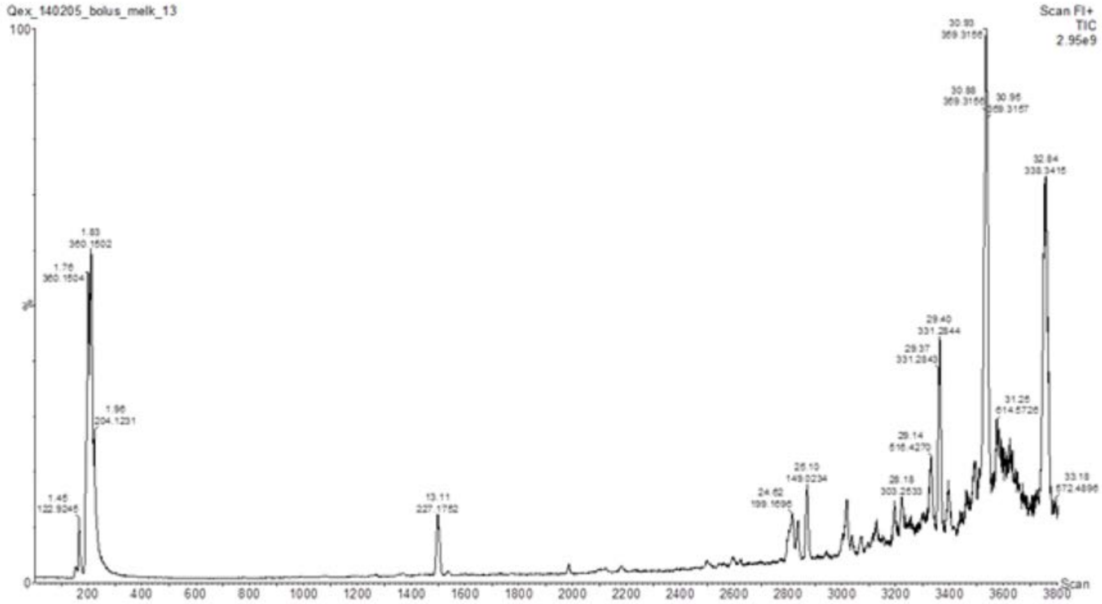
Annex 5 LC-MS spectra of bolus and milk

Spectrum bolus in milk

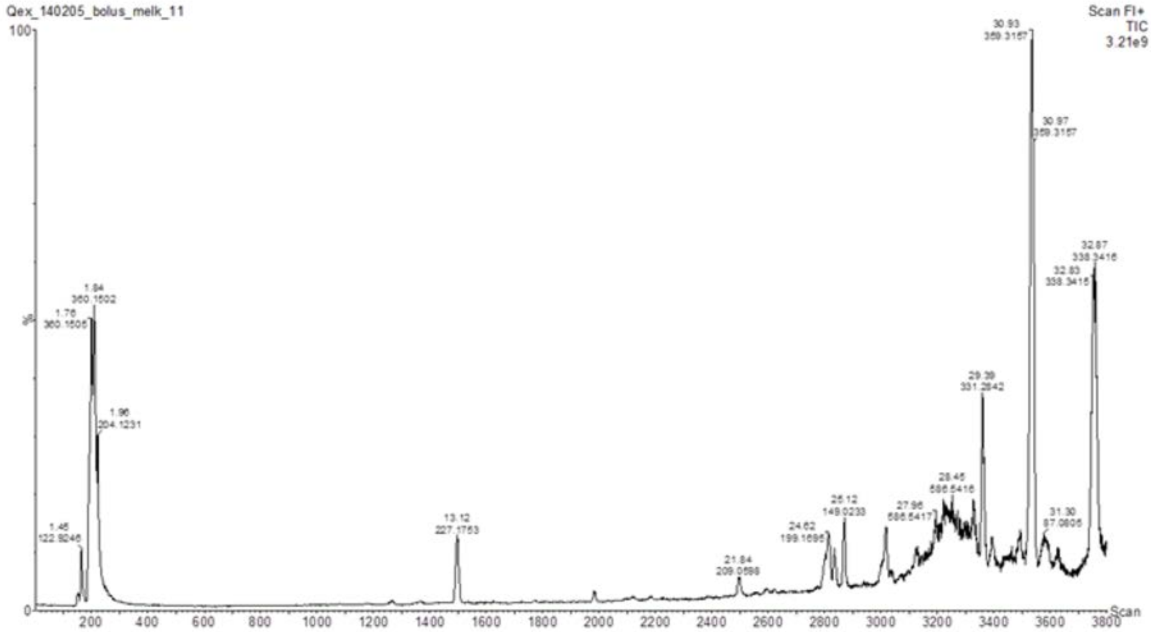




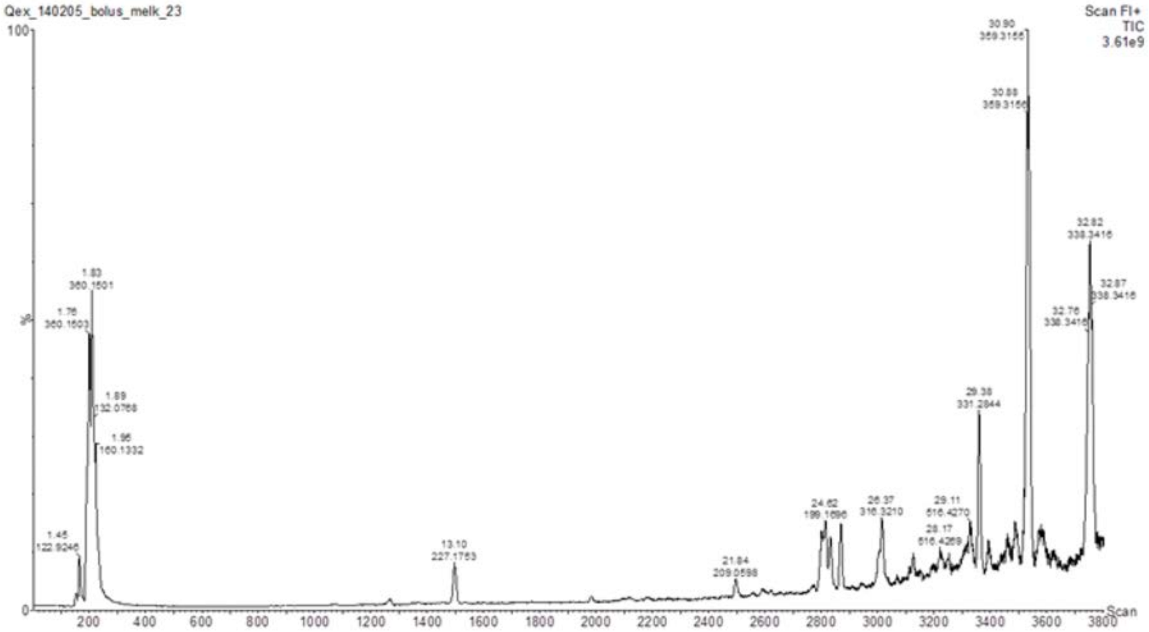
Cow 2376 day 0



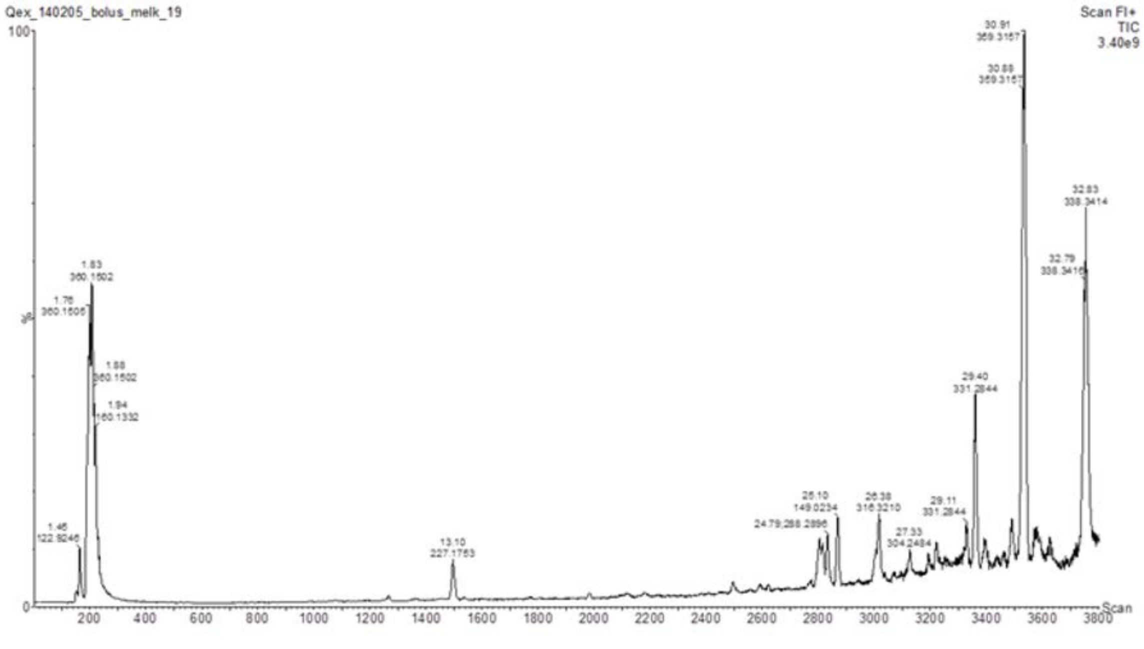
Cow 2376 day 1



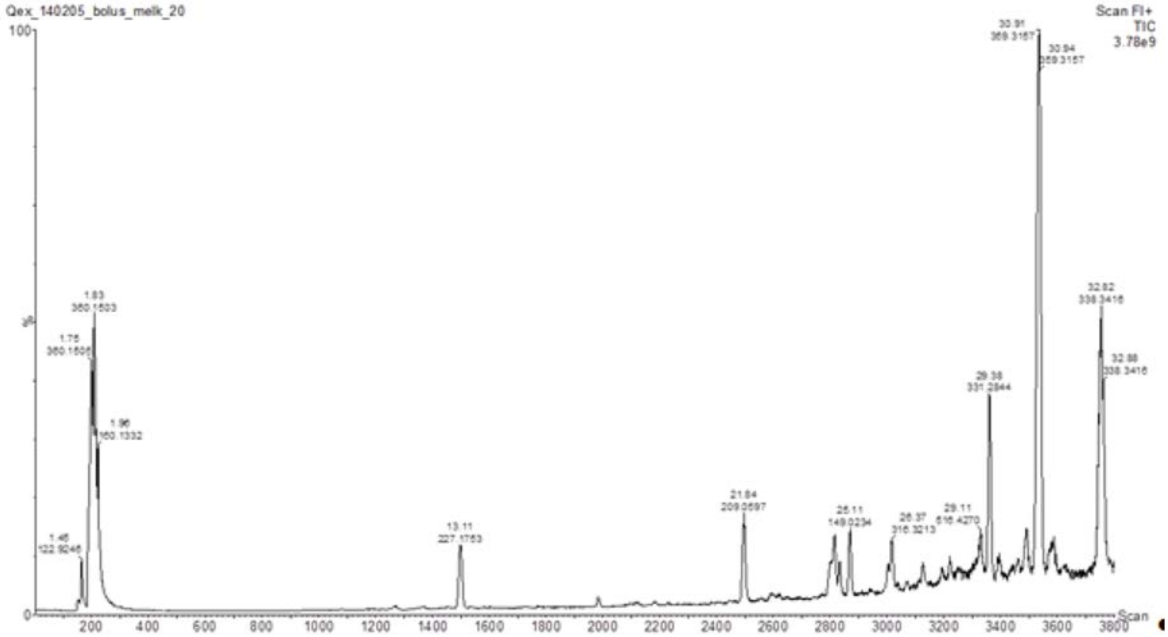
Cow 2376 day 3



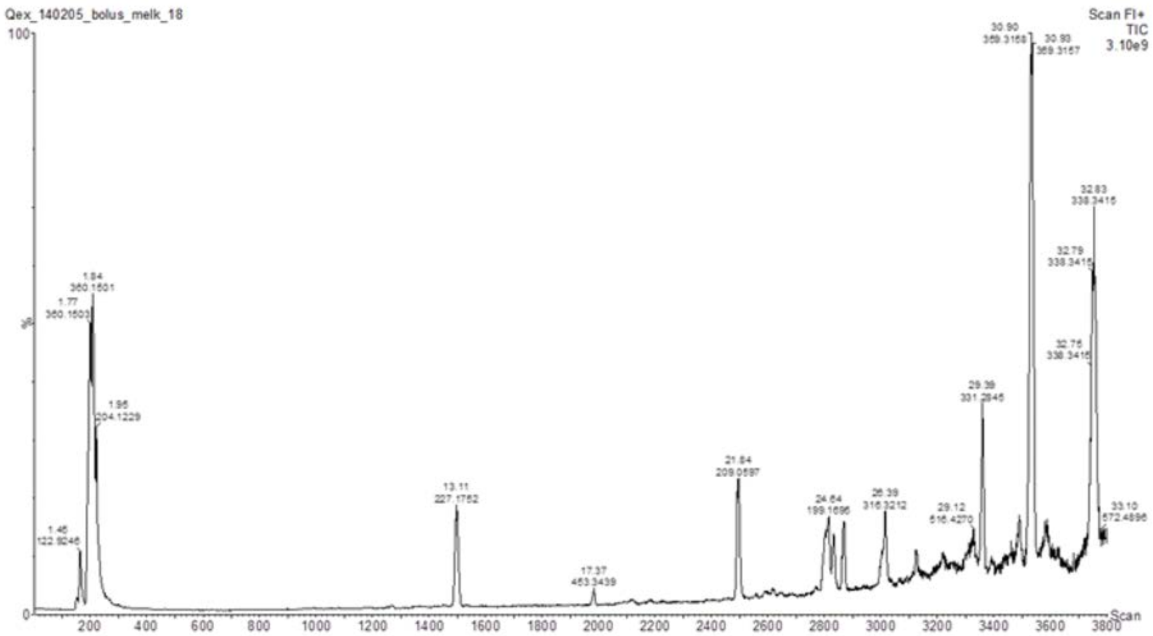
Cow 2376 day 7



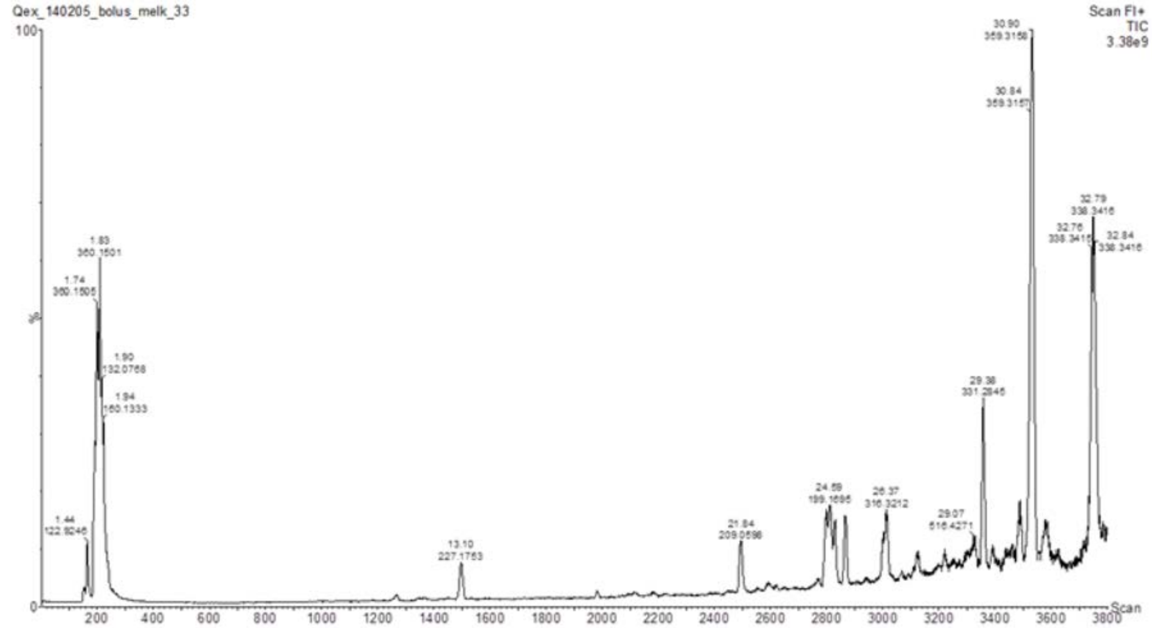
Cow 2173 day 0



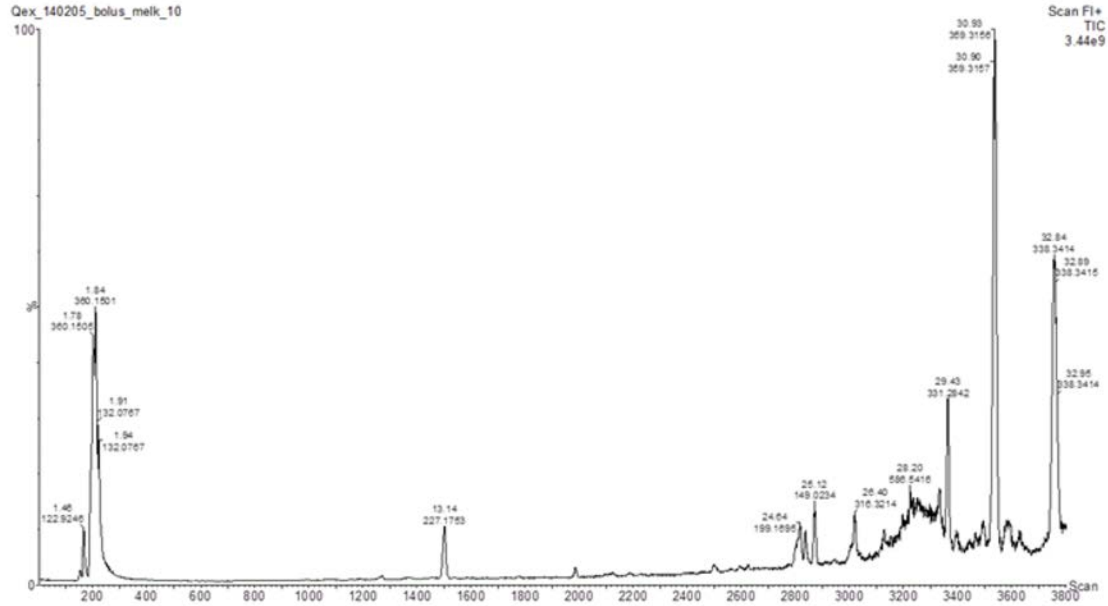
Cow 2173 day 1



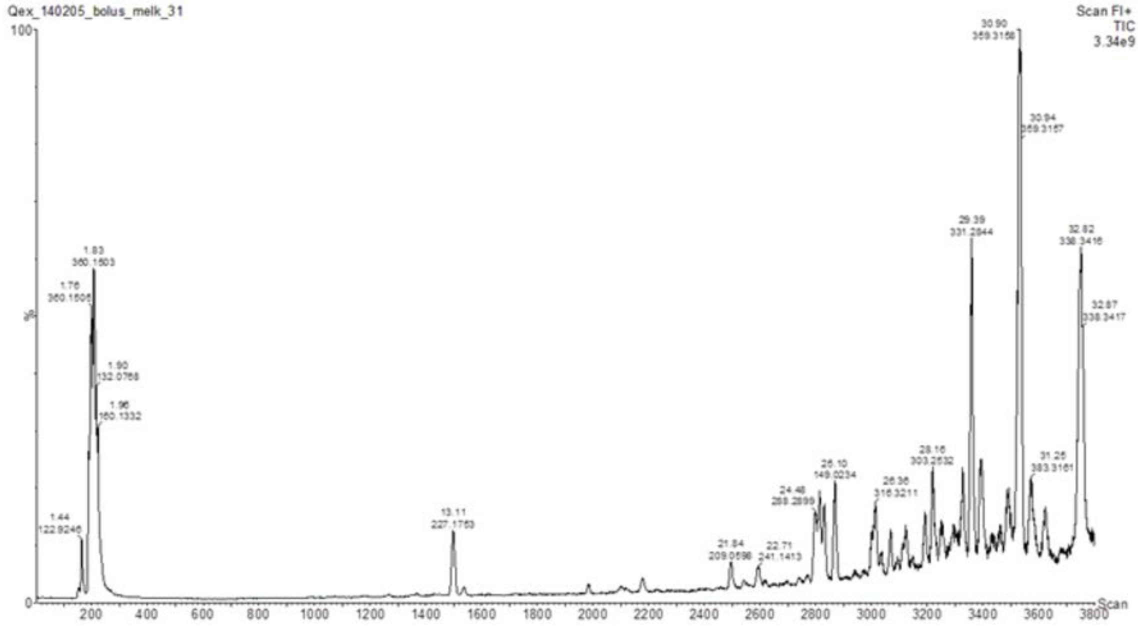
Cow 2173 day 3



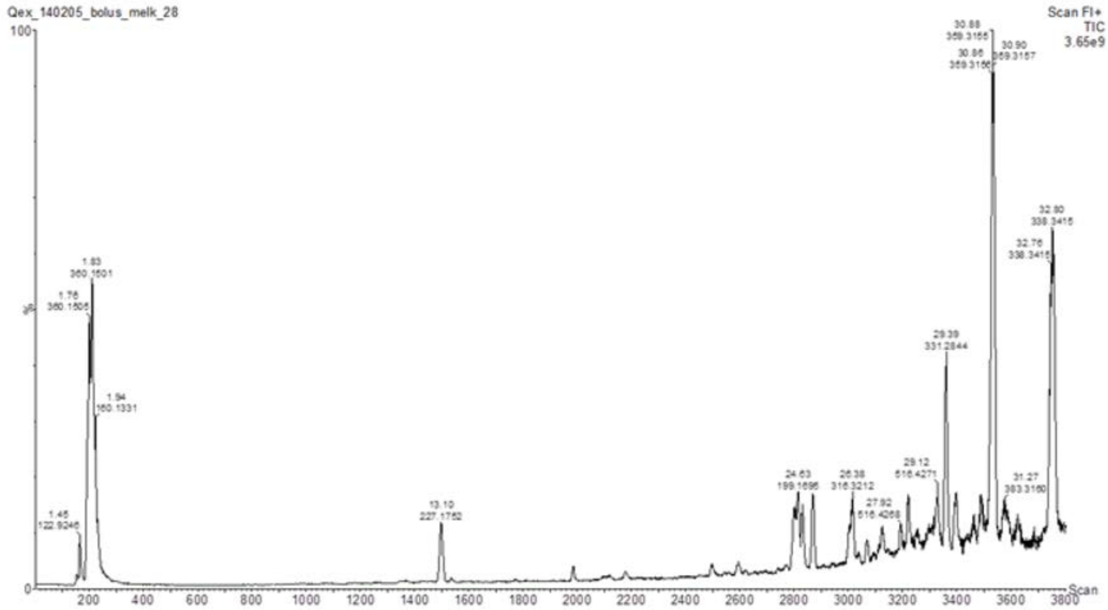
Cow 2173 day 7



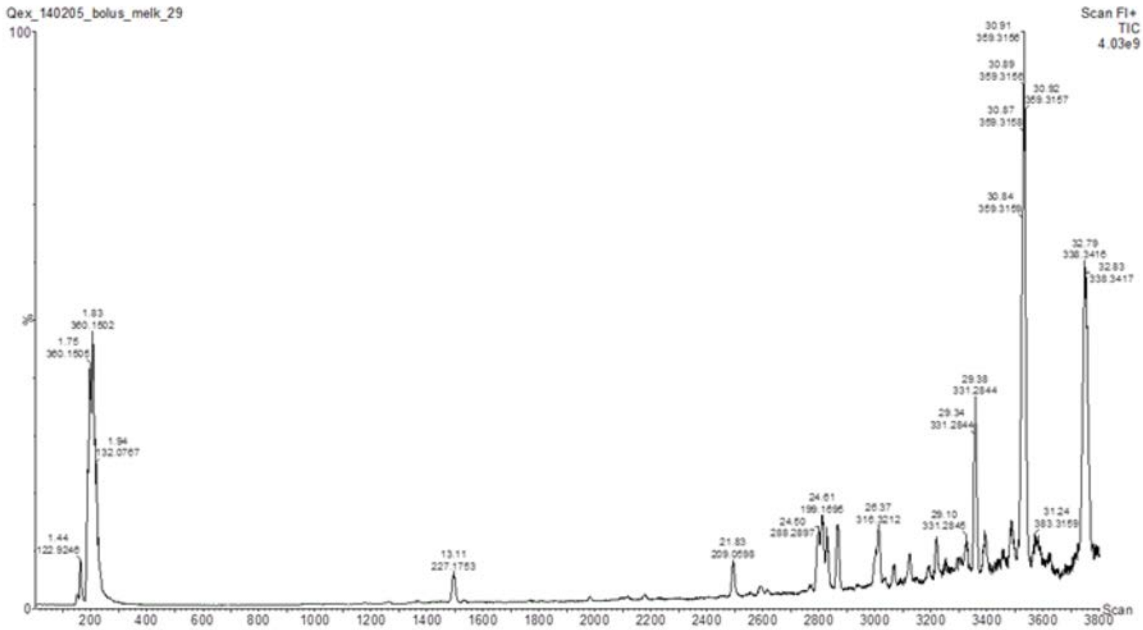
Cow 2150 day 0



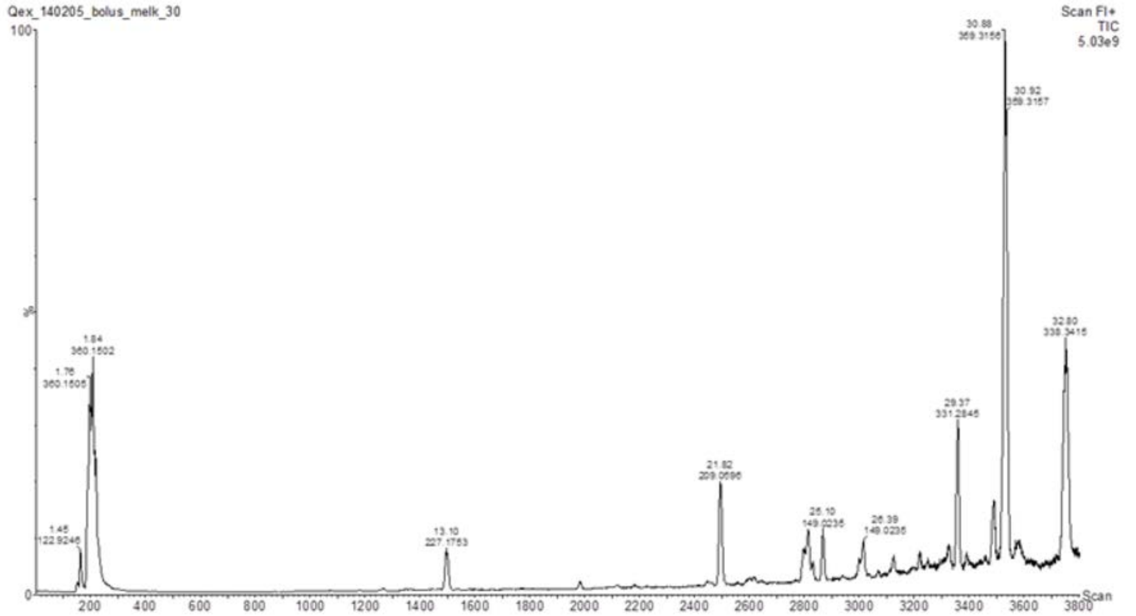
Cow 2150 day 1



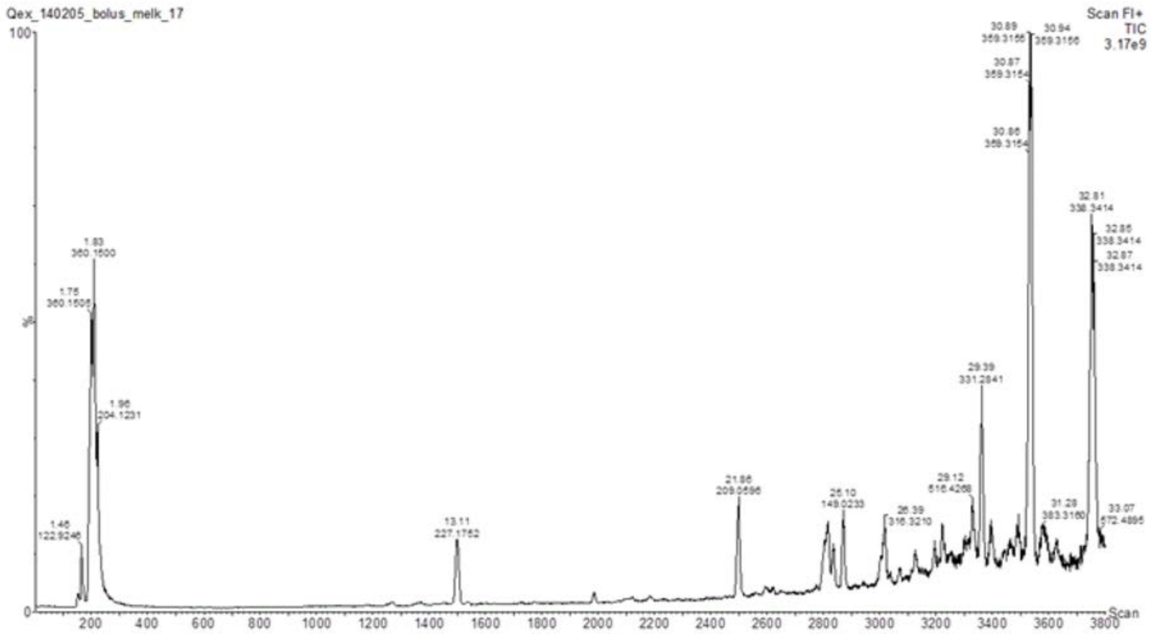
Cow 2150 day 3



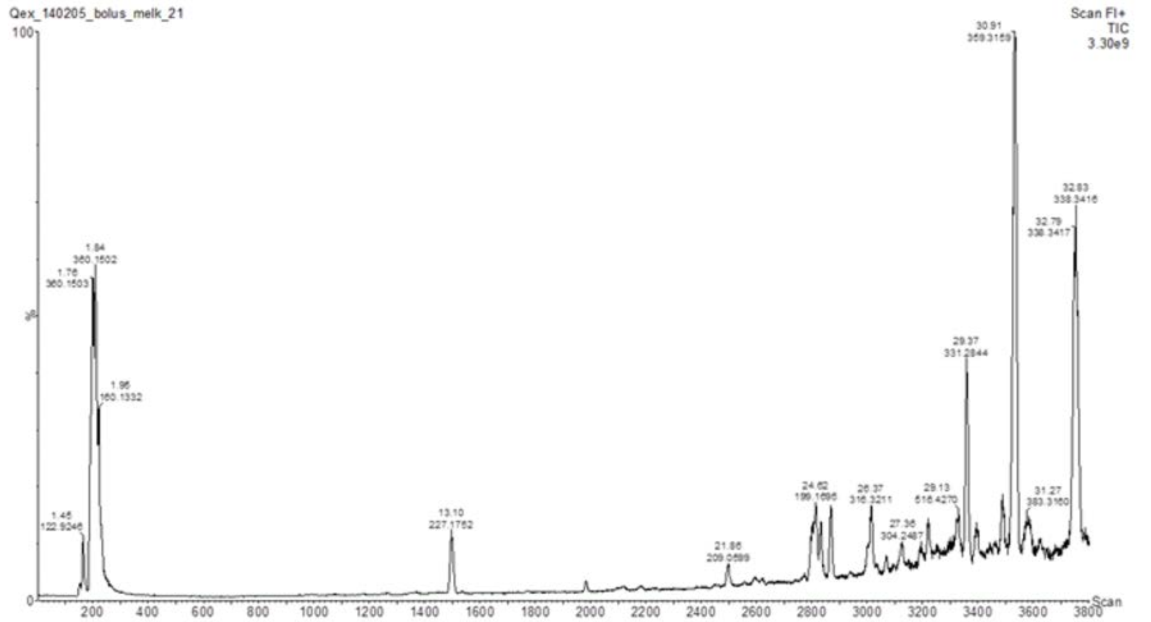
Cow 2150 day 7



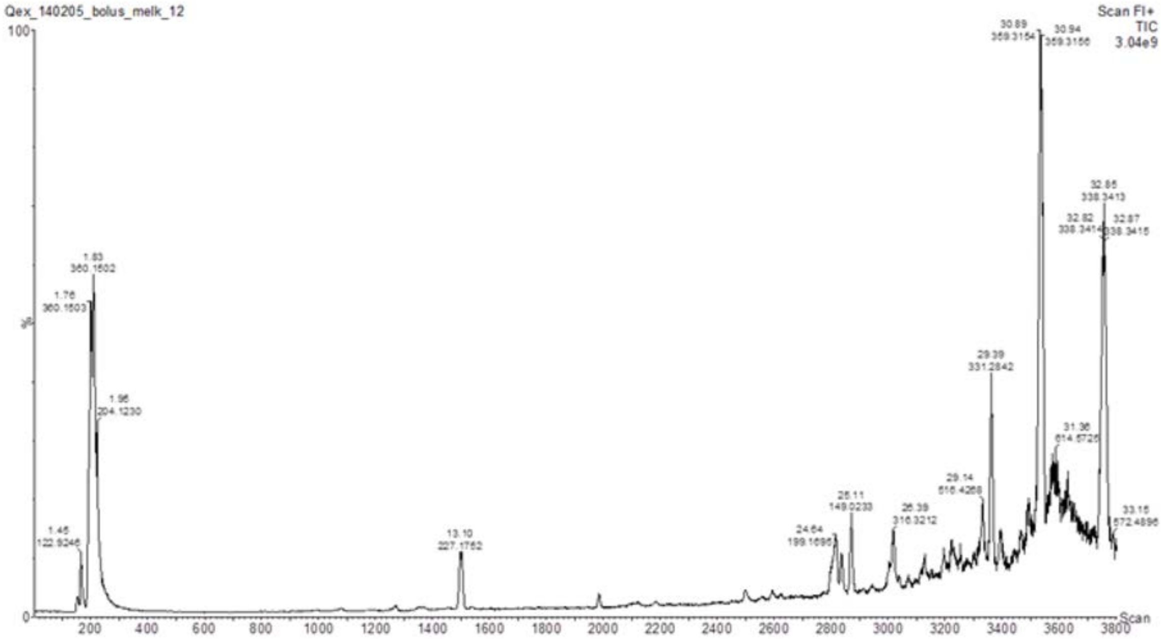
Cow 2317 day 0



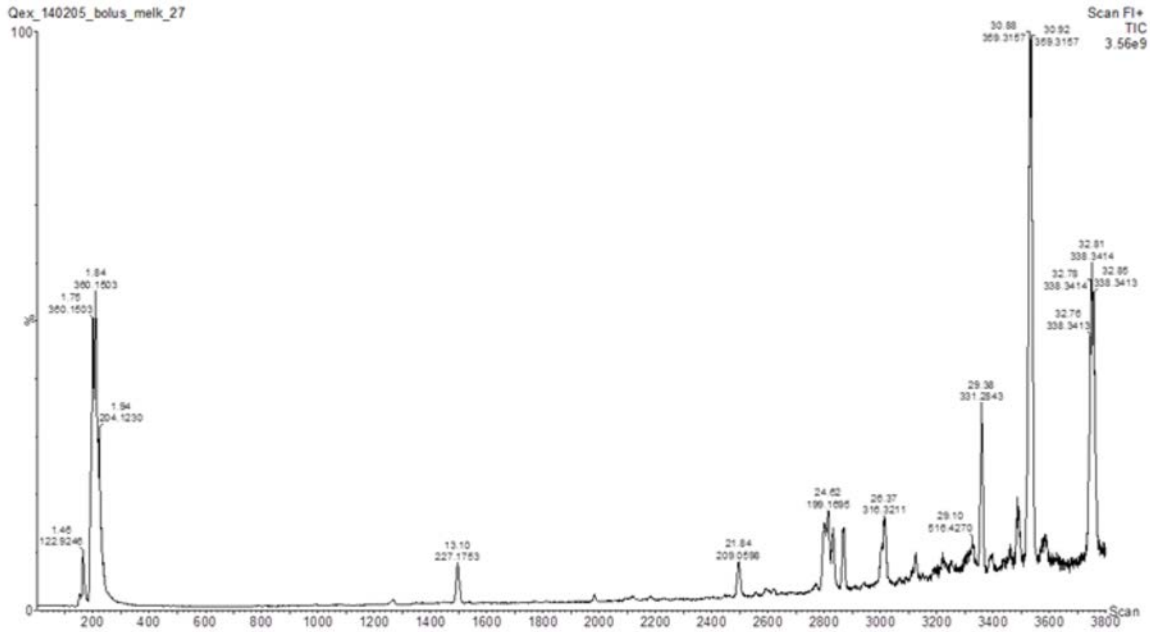
Cow 2317 day 1



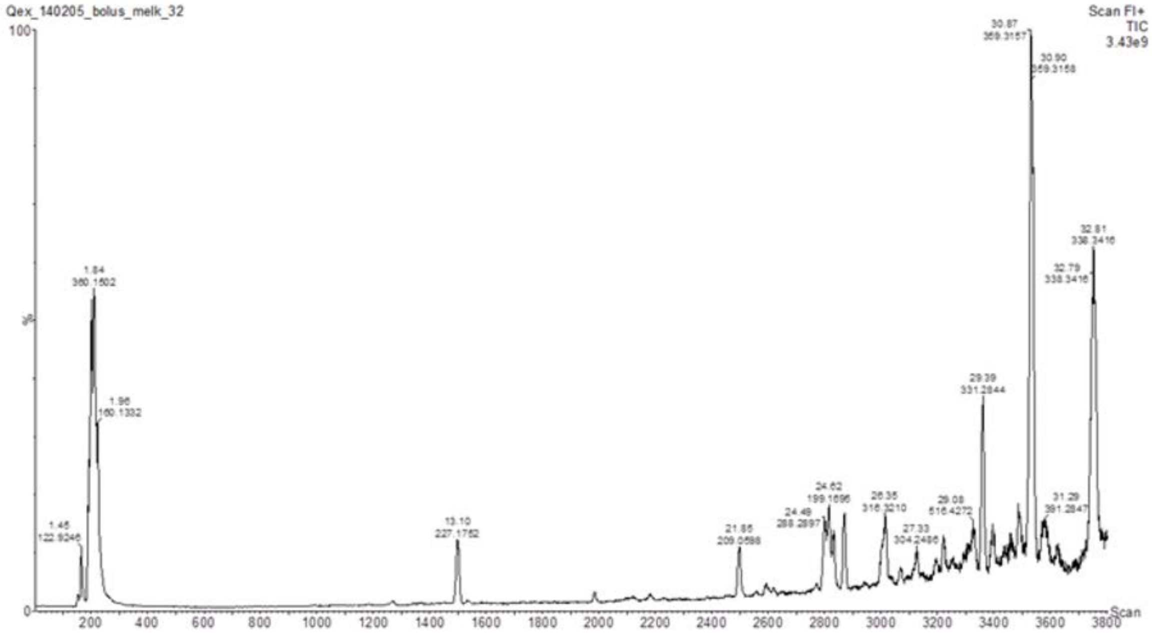
Cow 2317 day 3



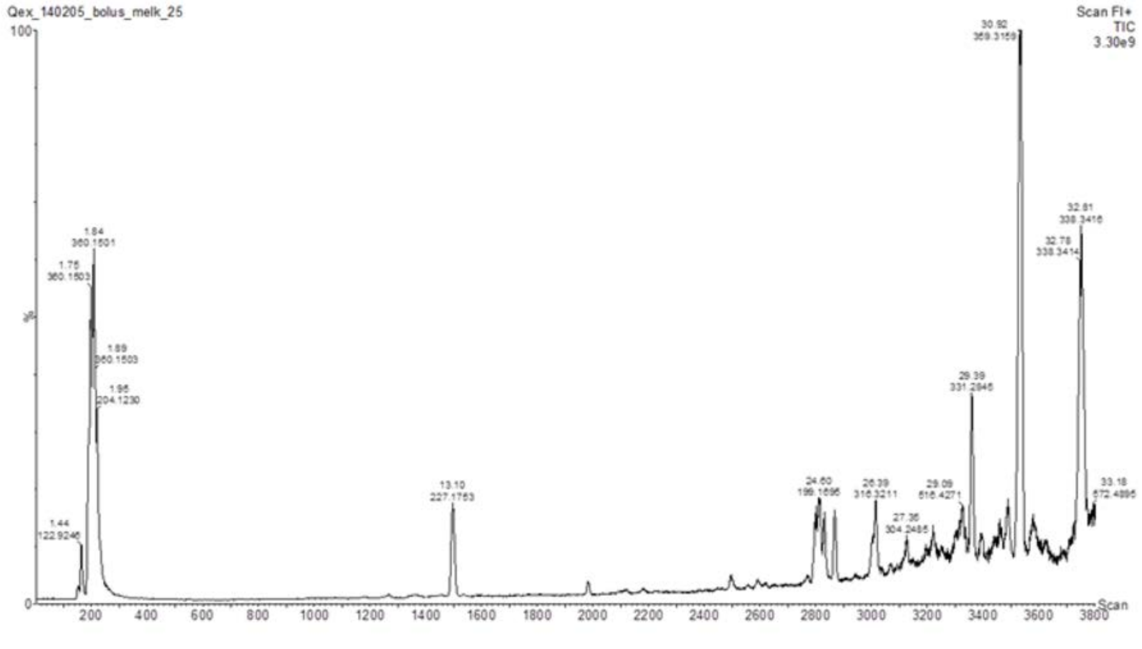
Cow 2317 day 7



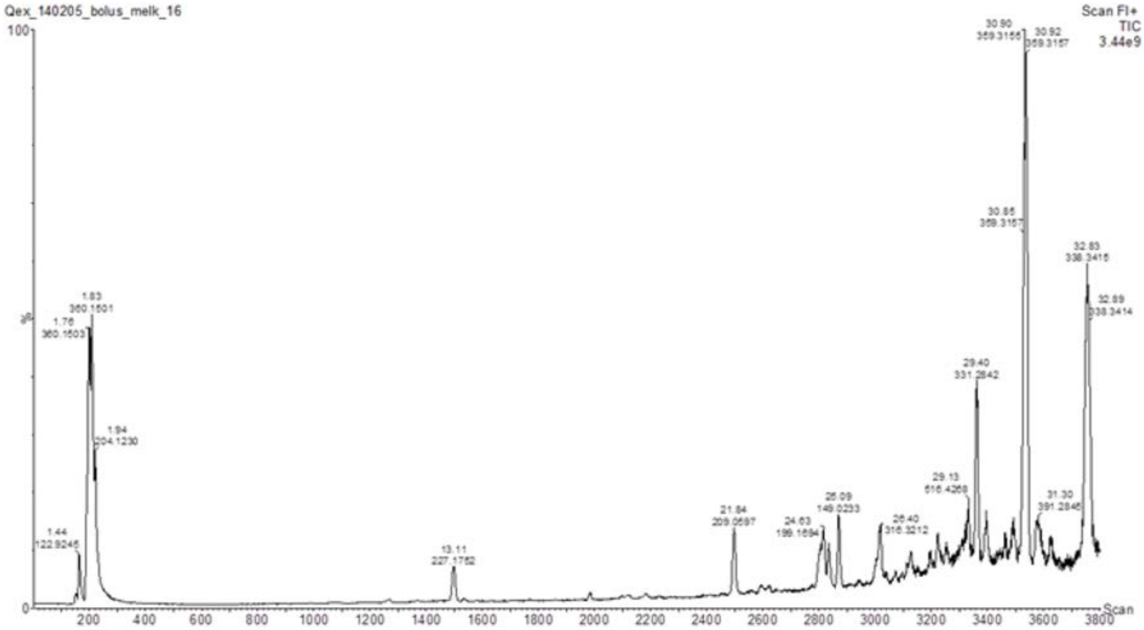
Cow 2106 day 0



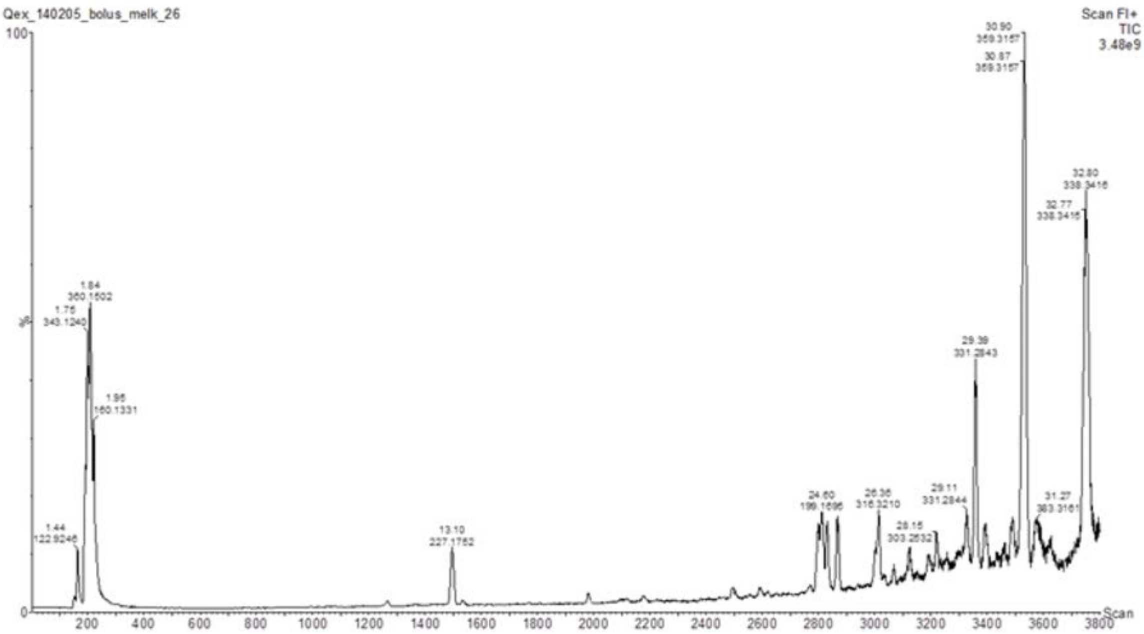
Cow 2106 day 1



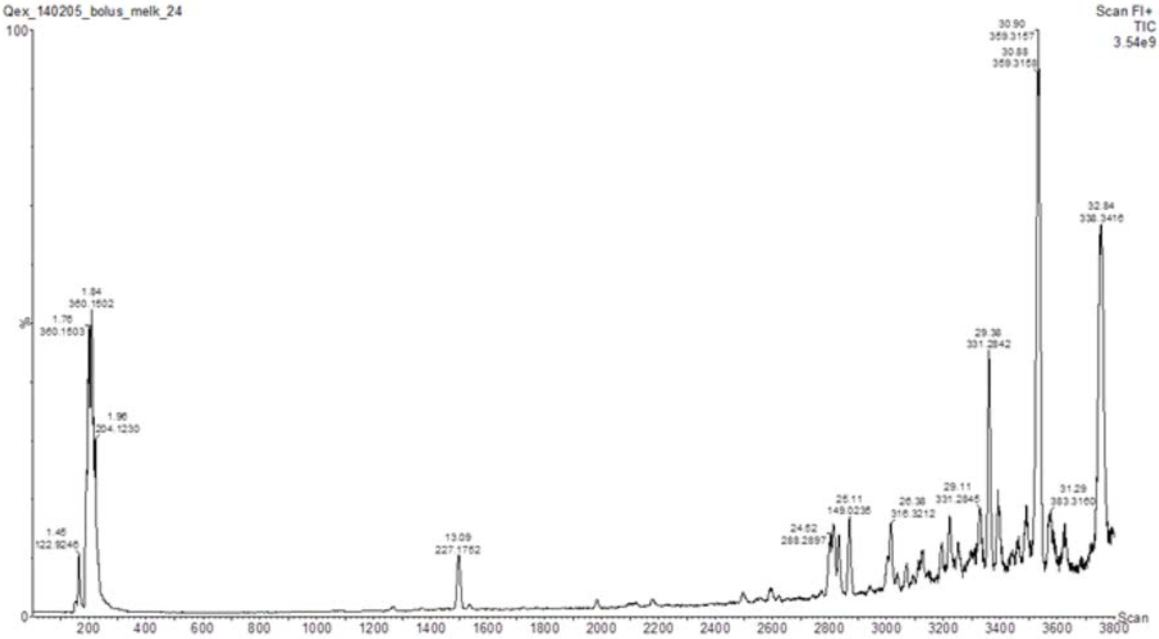
Cow 2106 day 3



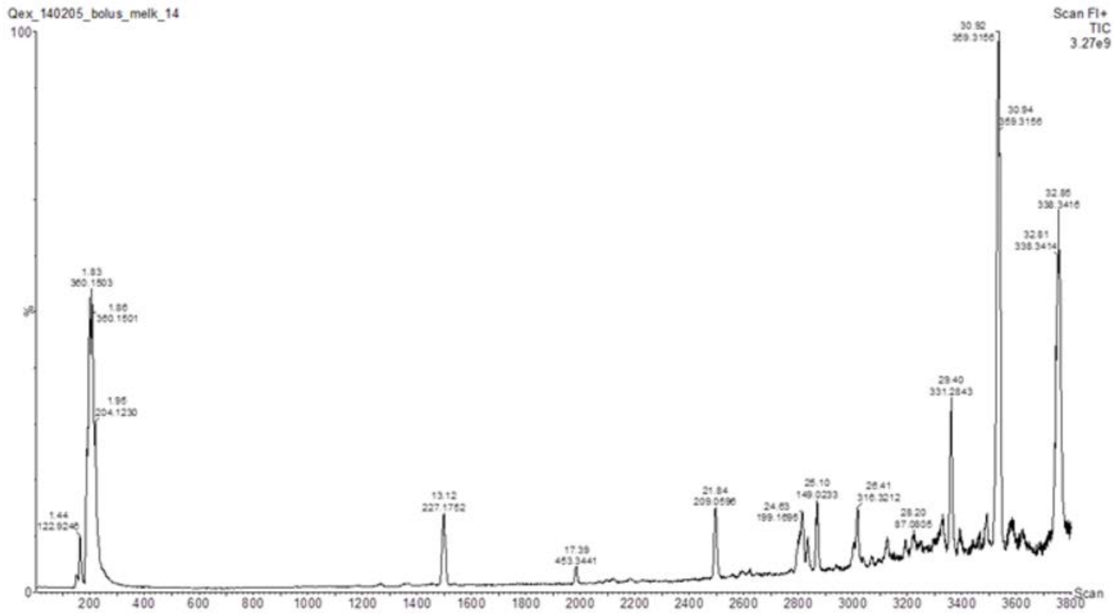
Cow 2106 day 7



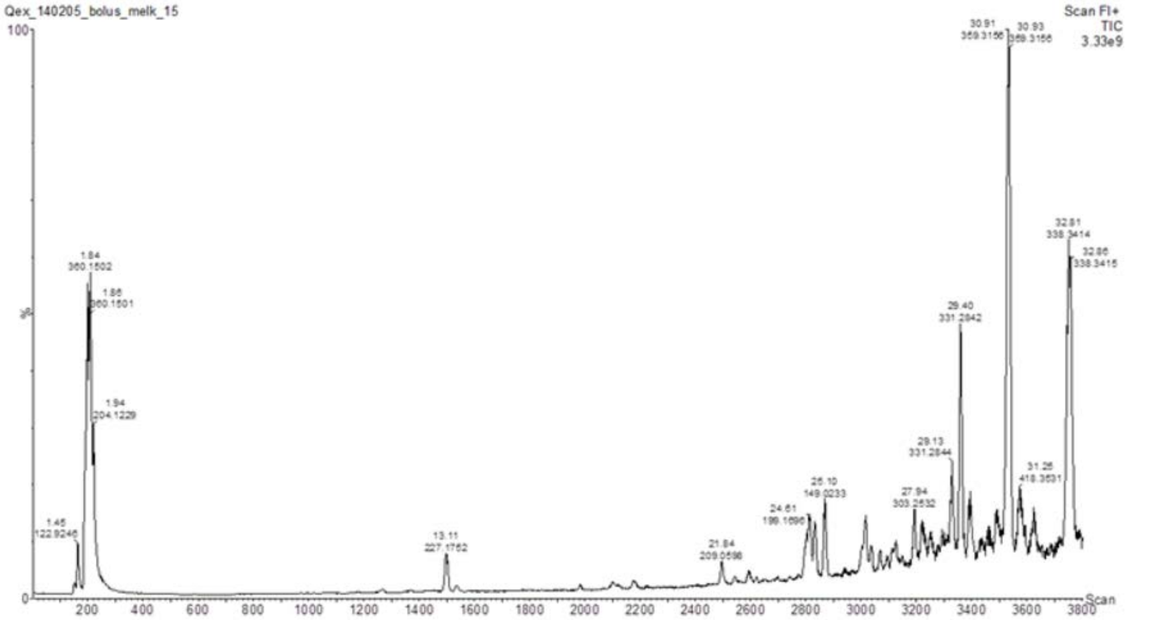
Cow 2320 day 0



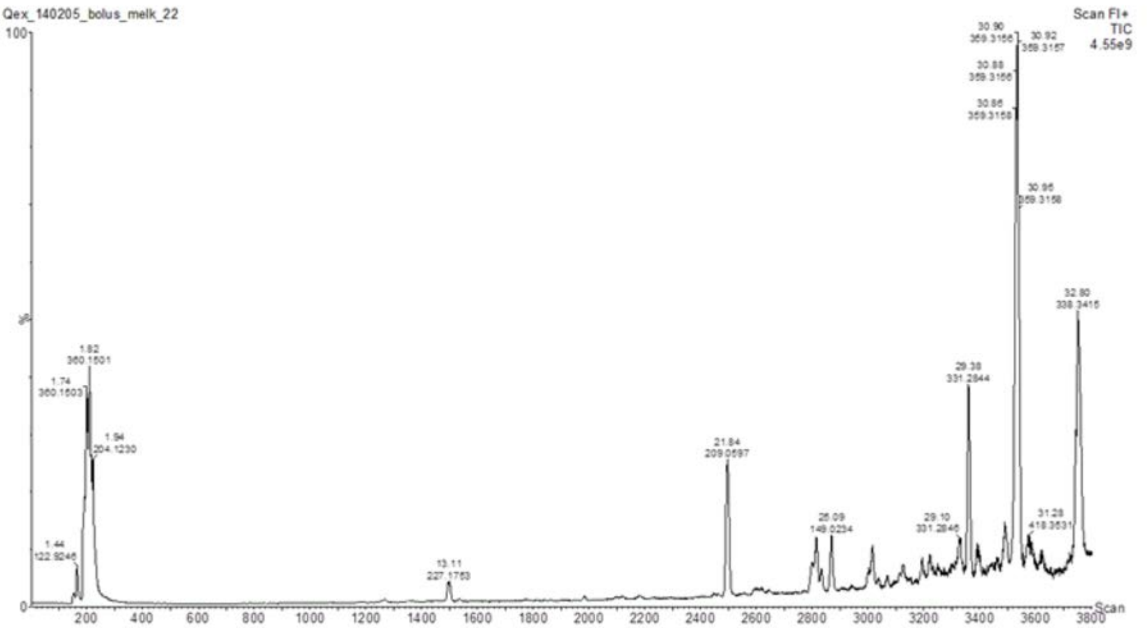
Cow 2320 day 1



Cow 2320 day 3



Cow 2320 day 7



RIKILT Wageningen UR
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0)317 48 02 56
www.wageningenUR.nl/en/rikilt

RIKILT report 2015.007

RIKILT Wageningen UR is part of the international knowledge organisation Wageningen University & Research centre. RIKILT conducts independent research into the safety and reliability of food. The institute is specialised in detecting and identifying substances in food and animal feed and determining the functionality and effect of those substances.

The mission of Wageningen UR (University & Research centre) is 'To explore the potential of nature to improve the quality of life'. Within Wageningen UR, nine specialised research institutes of the DLO Foundation have joined forces with Wageningen University to help answer the most important questions in the domain of healthy food and living environment. With approximately 30 locations, 6,000 members of staff and 9,000 students, Wageningen UR is one of the leading organisations in its domain worldwide. The integral approach to problems and the cooperation between the various disciplines are at the heart of the unique Wageningen Approach.

To explore
the potential
of nature to
improve the
quality of life

RIKILT Wageningen UR
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0)317 48 02 56
www.wageningenUR.nl/en/rikilt

RIKILT report 2015.007

RIKILT Wageningen UR is part of the international knowledge organisation Wageningen University & Research centre. RIKILT conducts independent research into the safety and reliability of food. The institute is specialised in detecting and identifying substances in food and animal feed and determining the functionality and effect of those substances.

The mission of Wageningen UR (University & Research centre) is 'To explore the potential of nature to improve the quality of life'. Within Wageningen UR, nine specialised research institutes of the DLO Foundation have joined forces with Wageningen University to help answer the most important questions in the domain of healthy food and living environment. With approximately 30 locations, 6,000 members of staff and 9,000 students, Wageningen UR is one of the leading organisations in its domain worldwide. The integral approach to problems and the cooperation between the various disciplines are at the heart of the unique Wageningen Approach.