Universitat Autònoma de Barcelona

Barcelona, March 20, 2017

DairyCare COST Action

Scientific Report for Short-term Scientific Mission (STSM)

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Period: 2017-02-20 to 2017-03-20
COST Action: FA1308
STSM type: Regular (from Italy to Spain)
STSM Applicant: Dr Valentina Caprarulo, University of Milan, Milan (IT), valentina.caprarulo@unimi.it
STSM Topic: 1H NMR spectroscopy technique as a valuable tool in metabolomics studies
Host: Ahmed A.K. Salama, Universitat Autonoma de Barcelona, Barcelona (ES), ahmed.salama@uab.cat

1. Purpose of the STSM:

Consistent with literature, several protocols have been used to prepare protein and lipid-containing biofluids such as serum, urine and milk for ^{1}H NMR profiling studies. These different protocols were adopted based on the type of biological fluid collected, and on the type of NMR analysis, specifically qualitative or quantitative analysis. In some protocols, filtration (de Graaf et al., 2003; Tiziani et al., 2008) or solvent extraction followed by drying steps (Tiziani et al., 2008) has been used to remove protein. However, both of these deproteination steps can result in variable loss of metabolites (Le Belle et al., 2002; Daykin et al., 2002). Furthermore, those protocols do not permit to study lipid-contain biofluids since only water/protein phase were analyzed by NMR.

In this respect, the aim of my STSM at the Univesitat Autonoma de Barcelona was to acquire new knowledge on the use of nuclear magnetic resonance. Specifically, the main objective was to develop and compare different sample preparation protocols in order to assess the most suitable method for ^{1}H NMR analysis.

2. Description of the work carried out during the STSM:

Different bio-fluid matrix namely, blood, milk, urine, rumen fluid and faeces were collected from ruminants in the experimental farm of the UAB. Specifically, blood, urine, faeces and milk samples were collected from goats; whereas rumen fluid was collected from fistulated cow. In order to obtain the best representative samples, a pool for each biological fluid was made from three different animals randomly selected. The analysis adopted 5 replicates for each biofluids.

Two different protocols (Figure 1) for samples preparation were adopted as follow:

- **UAB protocols**

- **TEST Protocol:** according to Sheedy and collaborators (2010), the best protocol was selected for the present experiment. Specifically, a Chloroform/Methanol extraction were adopted in order to well separate protein phase and lipid phase of different bio-fluid matrix namely, blood, milks, urine, rumen fluid and faeces, collected from ruminants.

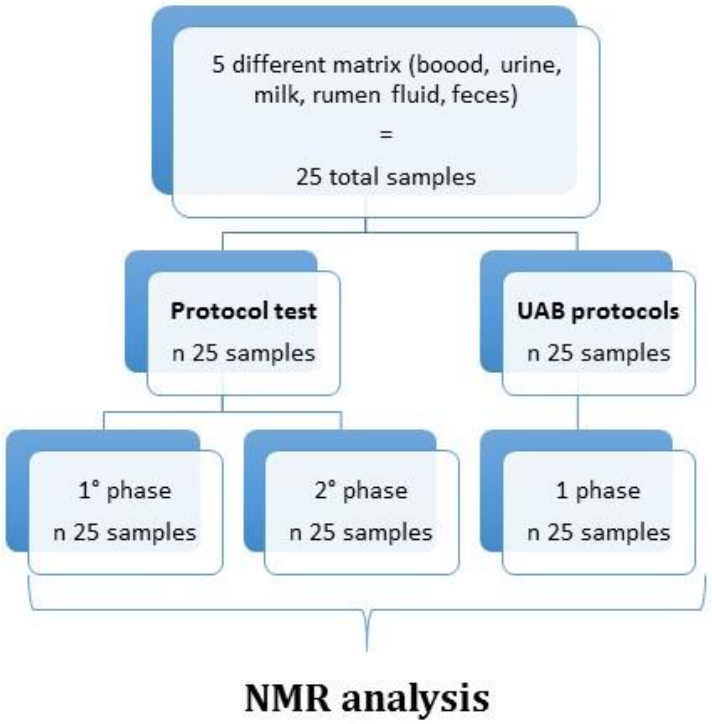

Figure 1. Flow chart of collected samples and adopted protocols.

Samples processed by UAB protocols or TEST protocol adopted the same phosphate buffer, in order to minimize differences related to reagents that can compromise the NRM analysis. The substantial difference between those two protocols consists in phase separation by Chloroform/methanol, which is adopted for the test protocol, but not for UAB protocols.

Samples preparation

Blood samples were collected from jugular vein (left and right) of dairy goats. Blood was centrifuged (4000 g, 4°C, 15 min) and the plasma from left and right jugular was pooled together. 1mL of serum, from three animals selected, was pooled in a 5mL Eppendorf tube. Following the UBA protocol (Figure 2), 200 µL of plasma was chilled on ice and diluted with chilled (350 µL) phosphate buffer (pH 7.4) in Eppendorf tube. Store a -20°C until NMR analysis. Following TEST protocol (adapted from Sheedy et al., 2010; Figure 2), 500 µL of plasma was chilled on ice and directly diluted with chilled deuterated chloroform (330 µL) and deuterated methanol (200 µL), and was incubated for 10 min on ice. The biphasic mixture was centrifuged (3000g, 4°C, 10 min), and aliquot of the upper phase (250 µL) was mixed with 300 µL phosphate buffer (pH 7.4), and

aliquot of the bottom phase (250 µL) was mixed with 300 µL deutered chloroform contained 1% (v/v) TMS. Store a -20°C until NMR analysis.

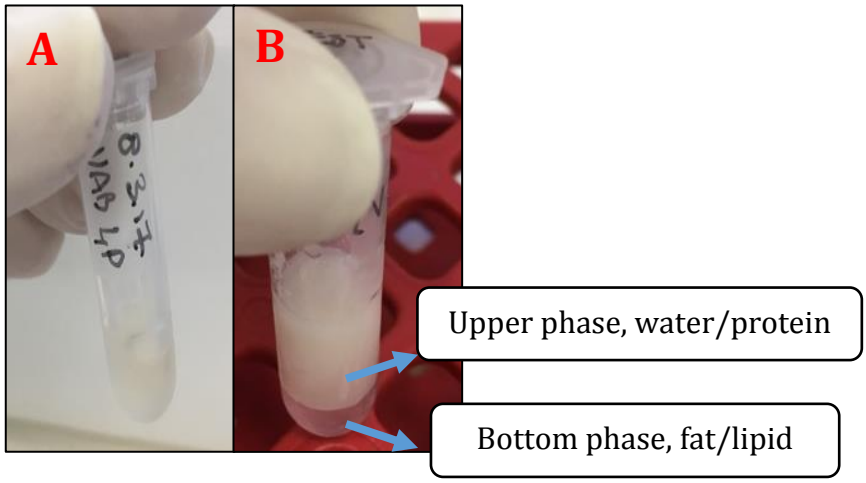

Figure 2. Plasma sample processed by UAB protocol (panel A) and sample processed by test protocol after centrifugation (panel B)

Urine samples were collected from dairy goats. 1.5 mL of urine, from three animals selected, was pooled in a 5mL Eppendorf tube. Following the UBA protocol , 350 µL of sample was chilled on ice and diluted with chilled (200 µL) phosphate buffer (pH 7.4) in Eppendorf tube. Store at -20°C until NMR analysis. Following TEST protocol (adapted from Sheedy et al., 2010; Figure 3), 500 µL of sample was chilled on ice and directly diluted with chilled deuterated chloroform (330 µL) and deuterated methanol (200 µL), and was incubated for 10 min on ice. The biphasic mixture was centrifuged (300g, 4°C, 10 min), and aliquot of the upper phase (250 µL) was mixed with 300 µL phosphate buffer (pH 7.4), and aliquot of the bottom phase (250 µL) was mixed with 300 µL deutered chloroform contained 1% (v/v) TMS. Store at -20°C until NMR analysis.

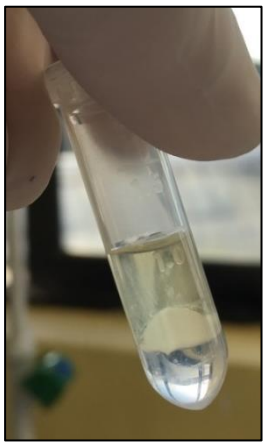

Figure 3. Urine sample processed by test protocol after centrifugation.

Faeces samples were collected from dairy goats. 2 g of faeces, from three animals selected, was pooled in 50 mL Eppendorf tube. Following the UBA protocol (adapted from Chai et al., 2015), 7

mL of phosphate buffer was added to 140 mg of fecal material, and vortex for 5 min. Fecal material was centrifuged (4000g, 4°C, 10 min). The upper phase was collected and filtered through 0.2- µm membrane filters. Filtrated solution (550 µL) was transferred to a 2 mL Eppendorf tube and store at -20°C until NMR analysis. Following TEST protocol (adapted from Sheedy et al., 2010 and Chai et al., 2015), 6 mL (Figure 4; 1:3 ratio) or 8 ml (Figure 4; 1:4 ratio) of Milli Q water was added to 3 g of fecal material and centrifuged (4000g, 4°C, 10 min). The supernatant was collected (500 µL) and chilled on ice, and directly diluted with chilled deuterated chloroform (330 µL) and deuterated methanol (200 µL), and was incubated for 10 min on ice. The biphasic mixture was centrifuged (3000g, 4°C, 10 min), and aliquot of the upper phase (250 µL) was mixed with 300 µL phosphate buffer (pH 7.4), and aliquot of the bottom phase (250 µL) was mixed with 300 µL deutered chloroform contained 1% (v/v) TMS. Store a -20°C until NMR analysis.

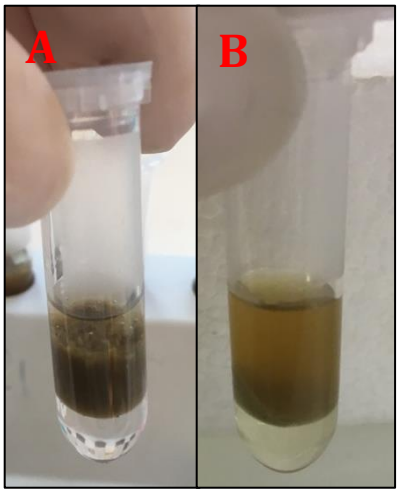

Figure 4. Samples with chloroform and methanol after centrifugation step, test protocol, 1:3 ratio (panel A) and 1:4 ratio (panel B)

Milk samples were collected from morning milking of dairy goats. Following the UBA protocol , 4 mL of milk was filtrated by Amicon Ultra-15 tubes (10 kDa cutoff) and centrifuge (5000g, 22°C, 20 min). Ultra-filtrated milk (350 µL) was mixed with 200 µL of cold buffer solution in Eppendorf tube. Store at -20°C until NMR analysis. Following TEST protocol (adapted from Sheedy et al., 2010), different sample and reagent ratio was tested. In TEST 0, 500 µL of milk was chilled on ice and directly diluted with chilled deuterated chloroform (300 µL) and deuterated methanol (200 µL); in TEST 1, 200 µL of milk was chilled on ice and directly diluted with chilled deuterated chloroform (300 µL) and deuterated methanol (200 µL); in TEST 2, 200 µL of milk was chilled on ice and directly diluted with chilled deuterated chloroform (400 µL) and deuterated methanol (250 µL); in TEST 3, 300 µL of milk was chilled on ice and directly diluted with chilled deuterated chloroform (450 µL) and deuterated methanol (300 µL); in TEST 4, 250 µL of milk was chilled on ice and directly diluted with chilled deuterated chloroform (450 µL) and deuterated methanol (300 µL). The best performance in term of phases' separation and amount of phases was TEST 4 (Figure 5), which was adopted to process milk samples. The biphasic mixture was centrifuged

(3000g, 4°C, 10 min), and aliquot of the upper phase (250 µL) was mixed with 300 µL phosphate buffer (pH 7.4), and aliquot of the bottom phase (250 µL) was mixed with 300 µL deutered chloroform contained 1% (v/v) TMS. Store a -20°C until NMR analysis.

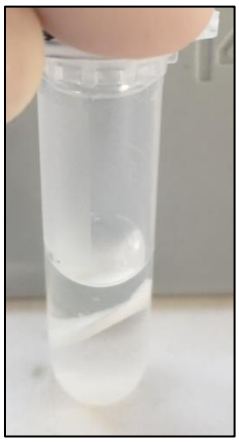

Figure 5. Chloroform and methanol extraction of milk samples using TEST 4.

Rumen fluid samples were collected from a fistulated dairy cow. Following the UBA protocol, 50 mL of rumen fluid was centrifuged (6000 g, 22°C, 15 min) and the supernatant was filtered through 0.2- µm membrane filters. Filtrated solution (350 µL) was mixed with 200 µL of cold phosphate buffer solution (pH 7.4) in an Eppendorf tube. Store at -20°C until NMR analysis. Following TEST protocol (adapted from Sheedy et al., 2010), 50 mL of rumen fluid was centrifuged (6000 g, 22°C, 15 min) and the supernatant (500 µL) was chilled on ice and directly diluted with chilled deuterated chloroform (300 µL) and deuterated methanol (200 µL). The biphasic mixture was centrifuged (3000g, 4°C, 10 min), and aliquot of the upper phase (250 µL) was mixed with 300 µL phosphate buffer (Figure 6; pH 7.4), and aliquot of the bottom phase (250 µL) was mixed with 300 µL deutered chloroform contained 1% (v/v) TMS. Store a -20°C until

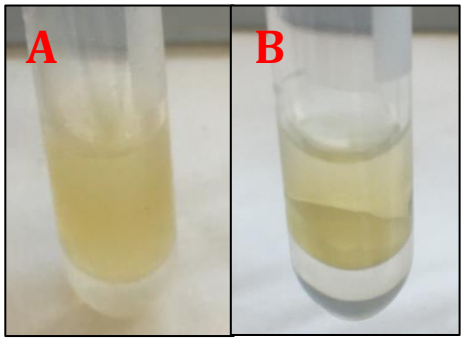

Figure 6. Rumen fluid sample with chloroform and methanol (panel A); rumen fluid sample after centrifugation step (panel B).

3. Description of the main results obtained:

The main objective was to develop and compare different sample preparation protocols in order to assess the more suitable method for ^{1}H NMR analysis. Adopting the TEST protocol, we were able to extract and analyze not only the water/protein phase but also the lipid containing phase.

In this respect adopting this procedure we will have a complete and more representative analysis of protein and lipid related metabolites from different biofluids matrix.

4. Future collaboration with the host institution (if applicable):

The collaboration with the Ruminant Research Group of Universitat Autonoma de Barcelona, will continue by the way of further NMR data analysis, paper preparation and with further activities. Samples processed during my stay at the Univesitat Autonoma de Barcelona will be analyzed by ^{1}H NMR, in order to obtain frequencies for each sample spectra of different biofluids matrix. The results obtained will be analyzed by the package "ChemoSpec" in the R software (version 3.2.3) and further analyzed by MetaboAnalyst 3.0. The STSM in Barcelona has been a valued experience to improve my skills and also to learn new lab procedures and sample preparation with regard to metabolomic techniques i.e. NMR.

5. Foreseen publications/articles resulting from the STSM (if applicable):

Results obtained from ^{1}H NMR will be processed by multivariate analyses and a manuscript will be prepared and sent for publication in a peer-reviewed journal.

6. Confirmation by the host institution of the successful execution of the STSM:

Confirmation letter from the host institution is attached to this report.

References

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