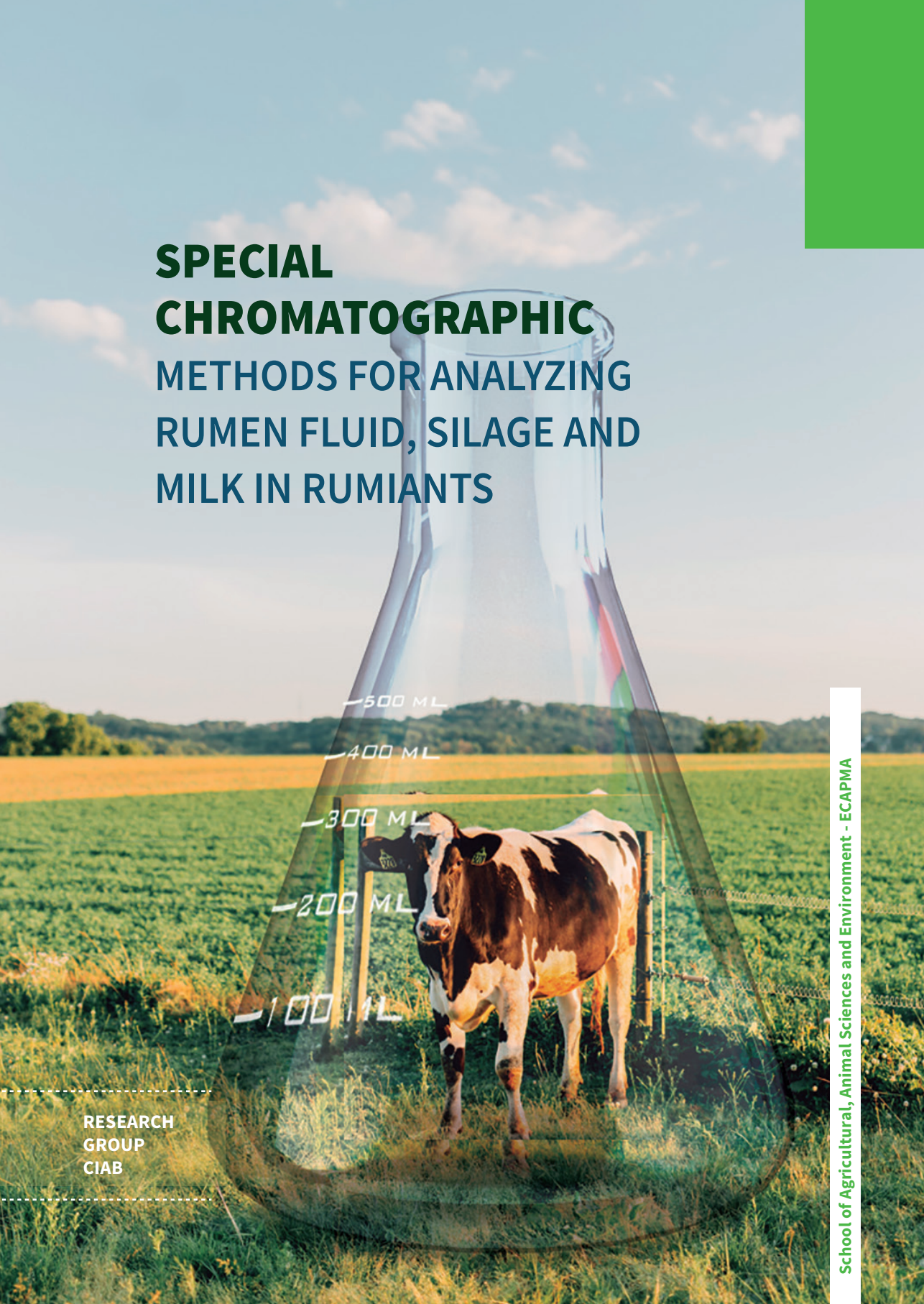
SPECIAL CHROMATOGRAPHIC METHODS FOR ANALYZING RUMEN FLUID, SILAGE AND MILK IN RUMIANTS



RESEARCH GROUP CIAB

School of Agricultural, Animal Sciences and Environment - ECAPMA

Sello Editorial
Universidad Nacional
Abierta y a Distancia

SPECIAL CHROMATOGRAPHIC METHODS FOR ANALYZING RUMEN FLUID, SILAGE, AND MILK IN RUMINANTS

Editor:
Julián Andrés Castillo Vargas

Research Group:
CIAB

UNIVERSIDAD NACIONAL ABIERTA Y A DISTANCIA – UNAD

Jaime Alberto Leal Afanador
Rector

Constanza Abadía García
Vicerrectora académica y de investigación

Leonardo Yunda Perlaza
Vicerrector de relaciones intersistémicas e internacionales

Édgar Guillermo Rodríguez Díaz
Vicerrector de servicios a aspirantes, estudiantes y egresados

Leonardo Evemeleth Sánchez Torres
Vicerrector de relaciones intersistémicas e internacionales

Julialba Ángel Osorio
Vicerrectora de inclusión social para el desarrollo regional y la proyección comunitaria

Myriam Leonor Torres
Decana Escuela de Ciencias de la Salud

Clara Esperanza Pedraza Goyeneche
Decana Escuela de Ciencias de la Educación

Alba Luz Serrano Rubiano
Decana Escuela de Ciencias Jurídicas y Políticas

Martha Viviana Vargas Galindo
Decana Escuela de Ciencias Sociales, Artes y Humanidades

Claudio Camilo González Clavijo
Decano Escuela de Ciencias Básicas, Tecnología e Ingeniería

Jordano Salamanca Bastidas
Decano Escuela de Ciencias Agrícolas, Pecuarias y del Medio Ambiente

Sandra Rocío Mondragón
Decana Escuela de Ciencias Administrativas, Contables, Económicas y de Negocios

SPECIAL CHROMATOGRAPHIC METHODS FOR ANALYZING RUMEN FLUID, SILAGE, AND MILK IN RUMINANTS

Editor: Julián Andrés Castillo Vargas

Grupo de Investigación: CIAB

541.372	**Castillo Vargas, Julián Andrés**
C352	Special chromatographic methods for analyzing rumen fluid, silage and milk in rumiants/ editor Julián Andrés Castillo Vargas -- [1.a. ed.]. Bogotá: Sello Editorial UNAD /2023. (Grupo de investigación CIAB)
	ISBN: 978-628-7857-26-1
	e-ISBN: 978-628-7857-27-8
	1. Métodos cromatográficos 2. Ciencias agrícolas 3. Investigaciones en farmacia 4. Análisis del líquido ruminal 5. Producción animal I. Castillo Vargas, Julián Andrés

ISBN: 978-628-7857-26-1

e-ISBN: 978-628-7857-27-8

School of Agricultural, Animal Sciences and Environment - ECAPMA

©Press

UNAD University Press

Universidad Nacional Abierta y a Distancia

Calle 14 sur No. 14-23

Bogotá D. C.

August 2026

Layout: Hipertexto - Netizen

How to cite this book: Castillo Vargas, J. (2026). *Special chromatographic methods for analyzing rumen fluid, silage and milk in rumiants*. UNAD University Press. **https://doi.org/10.22490/UNAD.9786287857278**

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivatives 4.0 Interna-tional License. **https://co.creativecommons.net/tipos-de-licencias/**

The continuous pursuit of maximizing ruminant production while minimizing its negative environmental impact has, in recent years, driven the development and implementation of various nutritional and feeding strategies.



BOOK REVIEW

The continuous pursuit of maximizing ruminant production while minimizing its negative environmental impact has, in recent years, driven the development and implementation of various nutritional and feeding strategies. As a result of this optimization, significant improvements have been achieved in both productive efficiency and compositional quality of ruminant-derived products.

The increasing demand to "produce more with less", **"produce sustainably,"** and **"enhance the nutritional quality"** of ruminant-derived products has, in recent years, required a stronger emphasis on transdisciplinarity. This means that agricultural sciences continuously seek support from related disciplines, including fundamental sciences such as chemistry, biology, geology, statistics, mathematics and pharmacy. Particularly chemical sciences provide essential tools for animal nutrition, enabling informed decision-making based on the composition of feed (e.g. forage, concentrates, by-products etc.), tissues (e.g. organs, muscles and bones) and ruminant-derived products (e.g. milk, meat and other food products). Thus, chemistry has the potential to support both the primary and the subsequent links in the livestock production chain.

In particular, instrumental analytical chemistry provides tools to understand the impact of adopted nutritional strategies or even the characteristics of ruminants on their nutrient metabolism and how this influences the quality of their products. In this context, modern instrumental methods, especially chromatographic and spectroscopic techniques, provide information in terms of the abundance and presence of organic compounds of interest, aiming to produce higher-quality food at a lower cost or with reduced environmental impact. However, specific books proposing the use of chromatographic techniques coupled with spectroscopic methods in the field of animal production are scarce and, at times, nonexistent.

Thus, this publication aims to provide a set of tools through the use of chemical-instrumental methods, focusing on the combination of chromatographic and spectroscopic techniques, seeking to offer diagnostic tools to optimize ruminant production. The material presents several methodologies for analyzing metabolites of interest in animal production in rumen fluid and ruminant milk, as well as silage, for this species.

AUTHORS' REVIEW



Julián Andrés Castillo Vargas

Holds a PhD in Animal Science from Universidade Estadual Paulista "Júlio de Mesquita Filho". Master's in animal production and bachelor's in chemistry from Universidad Nacional de Colombia. He is currently an active Postdoctoral student of the IIntegrated Postgraduate Program in Animal Science in the Tropics at the Universidade Federal do Norte do Tocantins and the Universidade Federal Rural da Amazônia. Contact: jcfcav@gmail.com

Tiago Costa de Araújo

Holds a degree in Natural Sciences with a specialization in chemistry from the Universidade do Estado do Pará. Currently serves as a Laboratory Technician in Chemistry at Universidade Federal Rural da Amazônia. Contact: tiago.araujo@ufra.edu.br

Vinícius da Silva Botelho Duarte Gomes

Master in Animal Science and Zootechnician from the Universidade Federal Rural da Amazônia. Currently works as a technical consultant at Premix Nutrição Animal. Contact: viniciusbotelhodg@gmail.com

Raylon Pereira Maciel

Master in Animal Science, and Zootechnician from the Universidade Federal do Tocantins. Currently works as a professor at the Universidade Federal Rural da Amazônia. Contact: raylonmaciel@gmail.com

Rafael Mezzomo

PhD and Master in Animal Science from Universidade Federal de Viçosa. Zootechnician from Universidade do Estado de Santa Catalina. Currently works as a professor at the Universidade Federal Rural da Amazônia. Contact: mezzomozoo@gmail.com

TABLE OF CONTENTS

PREFACE 13

Chapter 1

EXTRACTION, IDENTIFICATION AND QUANTIFICATION OF VOLATILE FATTY ACIDS (VFA) IN RUMEN FLUID SAMPLES USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN REVERSE PHASE COUPLED TO DIODE ARRAY DETECTOR (RP-HPLC-DAD) 17

1. Summary 18
2. Introduction 18
3. Reagents 19
4. Equipment 20
5. Procedure: 21
6. Calculations (for each VFA using the external standard method) 24
7. Analysis Time 25
8. Additional Comments 26
9. Acknowledgments 26
10. References 27

Chapter 2

PROTOCOL FOR THE EXTRACTION, IDENTIFICATION AND QUANTIFICATION OF VOLATILE FATTY ACIDS (VFAs) IN SILAGE USING REVERSE PHASE HIGH-EFFICIENCY LIQUID CHROMATOGRAPHY COUPLED TO DIODE ARRAY DETECTOR (RP-HPLC-DAD) 29

1. Summary 30
2. Introduction 30
3. Reagents 31
4. Equipment 33
5.Procedure 34
6. Calculations (for each VFA using the external standard method) 36
7. Analysis Time 38
8. Additional Comments 38
9. Acknowledgments 39
10. References 39

Chapter 3
EXTRACTION, IDENTIFICATION, AND QUANTIFICATION OF BUFFALO MILK PROTEINS USING THE COMBINATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC) AND NEAR INFRARED SPECTROSCOPY (NIRS) MEASUREMENTS 43

1. Summary 44
2. Introduction 44
3. Reagents 45
4. Equipment 46
5.Procedure 47
6. Calculations 50
7. Analysis Time 53
8. Additional Comments 53
10. Acknowledgments 54
11. References 54

Chapter 4
PROTOCOL FOR THE STUDY OF RUMINAL BIOHYDROGENATION OF UNSATURATED FATTY ACIDS FROM LIPID SUPPLEMENTS MIXED WITH FORAGE USING AN IN VITRO APPROACH 57

1. Summary 58
2. Introduction 58
3. Reagents 59
4. Equipment 60
5. Procedure 61
6. Analysis Time 68
7. Additional Comments 68
8. Acknowledgments 68
9. References 69

List of figures

Chapter 1

Figure 1. Chromatogram of a 10 mM standard mixture of VFAs. 24

Figure 2. Bovine rumen fluid sample chromatogram showing the identification of several VFAs. 25

Chapter 2

Figure 1.To do this, use the equipment's software and determine the area under each peak by integration. 37

Figure 2. Chromatogram of the actual sample of corn silage using the program presented in Table 2. Source: Chromeleon™ 7.2 Chromatography Data System (CDS) Software 38

Chapter 3

Figure 1. Chromatographic profiles of buffalo milk from different buffaloes (a, b, c and d). 50

Chapter 4

Figure 1. Tubes with rubber caps with a hole, inside the thermostatic bath to be used in vitro incubations. 62

Figure 2. Inoculum prepared for in vitro incubations. 63

Figure 3. Ice bath for incubation tubes after addition of 2% w/v $HgCl_2$ solution to stop fatty acid biohydrogenation. 64

Figure 4. Freeze-dried incubation contents. (a) Setup of the vials in the lyophilizer 65

Figure 5. Scheme for extraction and quantification of fatty acids in incubation systems. 67

List of tables

Chapter 1

Table 1. Reagent quantities to be used to prepare the calibration curve. 20

Table 2. Gradient used in chromatographic run (VFA Mix). 23

Table 3. Gradient used in the chromatographic run (acetic, propionic and butyric acids). 23

Chapter 2

Table 1. Reagents quantities to be used to prepare the VFA calibration curve (acetic acid, propionic acid, butyric acid and other VFAs in the VFA Mix). . 32

Table 2. Reagents quantities to be used to prepare the lactic acid calibration curve. 33

Table. 3. Gradient used in chromatographic run (lactic acid and VFA Mix). 35

Table. 4. Gradient used in the chromatographic run (lactic, acetic, propionic and butyric acids). 36

Chapter 3

Table 1. Elution gradient to be used in the chromatographic run. 48



Analytical chemistry aims to separate, identify and quantify analytes in various matrices and is based on the use of two major types of techniques: classical techniques and instrumental techniques.



PREFACE

Animal nutrition, as one of the branches of animal science, seeks to provide solutions for the producers in terms of maximizing animal production. To do so, animal science integrates knowledge from various fields, including analytical chemistry. This book emerges from the need to develop solutions in animal nutrition, aiming to establish a practical connection between analytical chemistry and animal science, to support decision-making in the latter field.

Analytical chemistry aims to separate, identify and quantify analytes in various matrices and is based on the use of two major types of techniques: classical techniques and instrumental techniques. Classical techniques, such as gravimetry and volumetry, are based on determining analyte concentration by measuring the mass (gravimetry) and volume (volumetry) of substances. These techniques are cost-effective and easy to implement; however, their main drawback is the inability to detect very low analyte concentrations.

On the other hand, there are the instrumental techniques, which, despite needing calibration standards and a greater technical knowledge than the classical techniques, offer the advantage of identifying and quantifying a wide range of analytes, even at very low concentrations. Among the main instrumental techniques, chromatography stands out as the foundation of the methods presented in this book.

In general terms, chromatography is a physicochemical technique for the separation, identification and quantification of substances, aimed at supporting decision-making in various fields of knowledge. There are two types of modern chromatographic techniques: high-performance liquid chromatography (HPLC) and gas chromatography (GC).

Both techniques make enable the separation of a mixture of substances dissolved in a liquid phase as well as the identification and quantification of their components. However, HPLC and GC are used to separate and identify low- and high-volatility substances, respectively.

In the chromatographic process, a solution of the mixture is injected into the chromatograph and the different substances are separated according to their physicochemical properties using a using a mobile phase–stationary phase system. Subsequently, the separated substances are identified through a spectroscopic detec-

In general terms, chromatography is a physicochemical technique for the separation, identification and quantification of substances, aimed at supporting decision-making in various fields of knowledge.

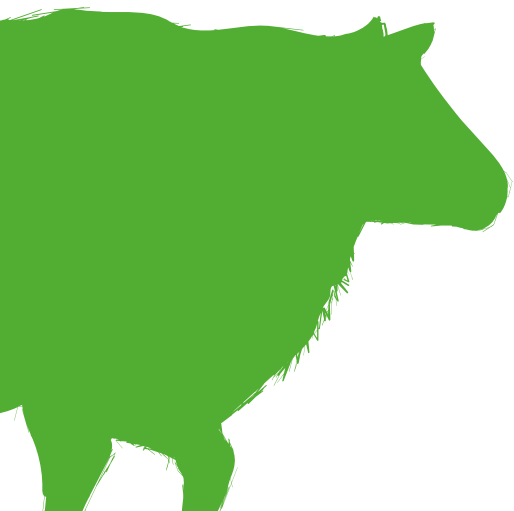
tion system, which involves a chemical reaction. Thus, this book aims to present the application of these chromatographic techniques in the development of methods for the separation, identification and quantification of substances involved in various contexts of ruminant nutrition.

The first chapter presents a rapid method for extracting, separating, and identifying volatile fatty acids (VFAs) in rumen fluid, which serve as powerful indicators of rumen digestibility in a diet. Chapter 2 focuses on VFAs determination in silage, key indicators of storage and production quality. Chapter 3 introduces a practical approach to quantitative determination of various protein fractions in casein and whey protein from buffalo milk, which provide essential elements to assess the nutritional quality of milk and the impacts of animal genetics on it. Finally, Chapter 4 outlines a comprehensive methodology for evaluating the rumen dynamics of long-chain unsaturated fatty acids, implementing the use of gas chromatography for their separation and quantification.

The proposed methods are derived from the authors' experience in the Special Analysis Laboratory of the Department of Animal Science at the Universidade Federal Rural da Amazônia and aim to support research in ruminant nutrition conducted by national and international researchers. The methodologies presented in this book, were developed with funding of the Programa Nacional de Cooperação Acadêmica na Amazônia (PROCAD/Amazônia, Proc. 88887.200488/201800), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superio (CAPES, Brasilia, DF, Brazil), the Conselho Nacional de Pesquisa e Desenvolvimento Científico e Tecnológico (CNPq), Fundação Amazônia de Amparo a Estudos e Pesquisa (FAPESPA), the Universidade Federal Rural da Amazônia (UFRA), the Research Division of Bogotá at the Universidad Nacional de Colombia and Colciencias (code: 1115-45221319). Additionally, this material is associated with the project: "Estudio del metabolismo ruminal de ácidos grasos poliinsaturados, con énfasis en la biosíntesis de precursores de lípidos bioactivos en leche y carne de rumiantes, usando análisis de correlaciones canónicas" (Proyecto PIE: ECAPMAPIE 132020)'.

Julián A. C. Vargas, PhD

Editor



The methodologies proposed in this book, were developed with funding of the Programa Nacional de Cooperação Acadêmica na Amazônia (PROCAD/ Amazônia) and the División de Investigación de Bogotá of the Universidad Nacional de Colombia






EZ
NL 6333
3721 4

EXTRACTION, IDENTIFICATION AND QUANTIFICATION OF VOLATILE FATTY ACIDS (VFA) IN RUMEN FLUID SAMPLES USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN REVERSE PHASE COUPLED TO DIODE ARRAY DETECTOR (RP-HPLC-DAD)



Julián Andrés Castillo Vargas
Universidad Nacional Abierta y a Distancia, Medellín, Antioquia, Colombia
Universidade Federal Rural da Amazônia, Parauapebas, Pará, Brasil
Universidade Federal do Norte do Tocantins, Araguaína, Tocantins, Brasil

Tiago Costa de Araújo
Universidade Federal Rural da Amazônia, Parauapebas, Pará, Brasil

Rafael Mezzomo
Universidade Federal Rural da Amazônia, Parauapebas, Pará, Brasil

1. Summary

In ruminant animals, volatile fatty acids (VFAs) also known as short-chain fatty acids (SCFAs) are derived from the fermentation of proteins and carbohydrates by ruminal microorganisms. Thus, VFA determination in rumen fluid allows for the evaluation of the nutritional quality of a diet, as well as its potential impact on the chemical composition of milk and meat from ruminants. Therefore, in this chapter, a protocol is presented for the extraction, identification, and quantification of the main VFAs in the rumen fluid, using reverse-phase high-performance liquid chromatography coupled with a diode array detector (RP-HPLC-DAD). The method involves the liquid-liquid extraction of VFAs from rumen fluid using 0.85% m/m phosphoric acid, with the extract analyzed by RP-HPLC-DAD after microfiltration. VFAs are quantified using the external standard method.

2. Introduction

Ruminants obtain nutrients from feed for the production of milk, meat and wool. However, nutrient utilization in feed involves various digestive processes, in which rumen microorganisms play a central role (Berchielli et al., 2011). When ruminants consume forage or concentrate, carbohydrate and protein molecules are initially catabolized by the action of enzymes secreted by bacteria, fungi and ruminal protozoa (Gomez-Insuasti et al., 2021). Most products of this catabolic process include volatile fatty acids (VFAs), most commonly acetic acid, propionic acid, and butyric acid. But others can be found, such as isobutyric acid, valeric acid, and caproic acid.

These VFAs are absorbed by the ruminal epithelium and converted through anabolic processes at the tissue level into carbohydrates, proteins and lipids, which are largely incorporated into milk and meat (Van Soest et al., 1994). Thus, the determination of VFA concentration in rumen fluid allows the assessment of the nutritional quality of a diet, as well as its potential impact on the chemical composition of milk and meat in ruminants (Kozloski, 2012).

When ruminants consume forage or concentrate, carbohydrate and protein molecules are initially catabolized by the action of enzymes secreted by bacteria, fungi and ruminal protozoa

With the development of chromatographic techniques, the discovery of new organic compounds, as well as the increased accuracy in determining the concentration of known ones, has increased in recent years (Ferlay et al., 2017).

Among these techniques, high-performance liquid chromatography (HPLC) stands out as a powerful method for the separation, identification and quantification of organic compounds in complex matrices, based on their physicochemical properties. Therefore, the objective of this chapter is to describe a rapid method for extracting, identifying and quantifying VFAs in rumen fluid samples, using RP-HPLC-DAD as the separation and quantification technique.

3. Reagents

3.1. Extraction solution

0.85% (w/w) orthophosphoric acid solution (working solution): carefully add 1 mL of 85% w/w orthophosphoric acid (Sigma Aldrich®) using a volumetric pipette to a 100 mL volumetric flask. Make up to 100 mL with distilled water.

3.2. Mobile phase components for chromatography analysis

3.2.1 *Phase A: 10 mM sodium phosphate buffer solution (pH = 2.6) (1 L):* weigh 0.78 g of sodium dihydrogen phosphate dihydrate (Sigma Aldrich®) and mix it with 0.34 mL of 85% v/v orthophosphoric acid in a beaker. Add 10 mL of Milli-Q® water to the mixture and transfer it to a 1L Class A volumetric flask. Make up to 1L with Milli-Q® water.

3.2.2. *Phase B: Acetonitrile HPLC grade* (Sigma Aldrich ®).

Calibration curve preparation for VFA quantification using the external standard method.

3.3.1 *Preparation of the calibration curve* in the VFAs concentration range *2 to 10 mM using a VFAs commercial mixture of* (CRM46975, Sigma Aldrich ®*):* considering that each VFA concentration in the mixture is equal to 10 mM, prepare 6 chromatographic vials and add to each one the reagent quantities described in Table 1 to prepare the calibration curve within the aforementioned concentration range:

Table 1. Reagent quantities to be used to prepare the calibration curve. Source: Authors

Level	Final concentration for each VFA (mM)	Commercial VFAs mix volume (µL)	Milli-Q ® water volume (µL)
1	2	200	800
2	4	400	600
3	5	500	500
4	6	600	400
5	8	800	200
6	10	1000	0

Notes:

a. Total volume for each calibration point is 1000 µL.

b. The calibration curves for each fatty acid can also be obtained using the individual VFAs. The commercial VFAs mix is suggested for operational practicality.

4. Equipment

a. HPLC equipped with self-sampler, Hypersil GOLD TM C18 column, Hypersil GOLD TM C18 pre-column, oven, and diode array detector.

b. Centrifuge (capable of reaching a minimum of 4,000 rpm).

c. Freezer (minimum temperature -20 °C).

d. Analytical balance.

e. Centrifuge tubes with a capacity of at least 5 mL.

f. Micropipettes from 100 µL to 5 mL and their tips.

g. Syringe and membrane filter (PES membrane), Whatman® brand, 0.45 µm.

h. 5 mL plastic syringes.

i. 1,5-2,0 mL chromatographic vials (amber) with septum and cap.

5. Procedure:

5.1. Collection of rumen fluid and sample preparation

5.1.1. From a fistulated ruminant (bovine, ovine or caprine), collect at least 50 mL of rumen fluid and filter it through three layers of gauze. Collect in a thermos preheated to 39 °C and transport the fluid to the laboratory as quickly as possible.

Notes:

a. For preheating the thermos, fill it with preheated water at 39°C and empty it at the time of collecting the rumen fluid.

b. The rumen fluid sample must be obtained from composite sampling of rumen fluid from three rumen regions: frontal, half ventral sac and cranial sac (Zijderveld et al., 2011).

5.1.2. In the laboratory, add 2 mL of pre-filtered rumen fluid to a 5 mL centrifuge tube.

5.1.3. Add 2 mL of extraction solution (see section 3.1) to the centrifuge tube containing the rumen fluid sample and shake it using a vortex mixer at medium speed for 30 s.

5.1.4. Freeze the sample at -20 °C until analysis.

Note:

If the sample is to be analyzed immediately after collection, refrigerate the tube to 4 °C for 30 min to allow protein precipitation in the rumen fluid.

5.2. Extraction of volatile fatty acids from rumen fluid



5.2.1. On the day of analysis, thaw the sample at room temperature (20 °C) and in a controlled manner.

5.2.2. Centrifuge the samples at 4,000 rpm for 10 minutes at room temperature (20°C).

5.2.3. Recover the liquid phase after centrifugation using a plastic syringe and filter the liquid phase through a 0,45 µm PES membrane filter. Collect the volume in a 4 mL plastic tube.

5.3. Chromatographic analysis

5.3.1. Chromatographic analysis of the calibration curve

5.3.1. Prepare the calibration curve as described in section 3.3.1 of reagents.

5.3.1.2. Analyze each point of the curve by RP-HPLC-DAD.

5.3.1.3. Identify and integrate the resulting chromatographic peaks.

5.3.2. Chromatographic analysis of rumen fluid samples

5.3.2.1. Place 500 µL of the final extract in a chromatographic vial.

5.3.2.2. Add 500 µL of Milli-Q® water.

5.3.2.3. Analyze by RP-HPLC-DAD.

5.3.2.4. Identify and integrate the resulting chromatographic peaks.

5.3.3. Chromatographic conditions

a. Column temperature: 30°C.

b. Injection volume: 20 µL.

c. Stationary phase: Hypersil GOLD™ C18 column (dimensions: 150 x 4.6 mm) equipped with Hypersil GOLD™ C18 guard column (dimensions: 10 x 4 mm).

d. Working wavelength: 210 nm.

e. Mode: gradient, constant flow: 0.750 mL/min (Table 2).



Table 2. Gradient used in chromatographic run (VFA Mix). Source: Authors.

Time (min)	Flow (mL/min)	Phase A (Phosphate buffer)	Phase B (Acetonitrile)
Equilíbrio			
-2,000	0,750	95	5
Corrida cromatográfica			
0,000	0,750	95	5
6,500	0,750	60	40
16,500	0,750	60	40
16,501	0,750	60	40
18,000	0,750	95	5

Notes:

a. If the goal is to separate and quantify only acetic, propionic and butyric acids (the most relevant VFAs in ruminal dynamics), the time of the chromatographic run can be shorter, and it is suggested to use the program described in table 3.

b. Tables 2 and 3 show a "negative" time. This corresponds to an equilibration time required to perform the chromatographic analysis. In the software of the equipment used, it should be inserted in this manner.

Table 3. Gradient used in the chromatographic run (acetic, propionic and butyric acids). Source: Authors.

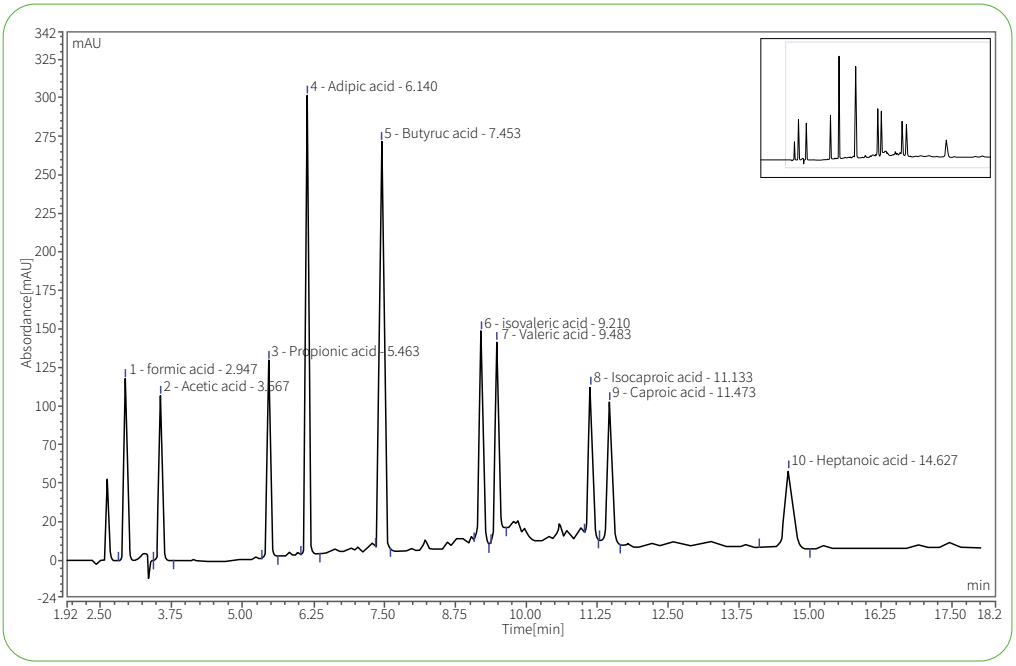
Time (min)	Flow (mL/min)	Phase A (Phosphate buffer)	Phase B (Acetonitrile)
Equilíbrio			
-2,000	0,750	95	5
Corrida cromatográfica			
0,000	0,750	95	5
6,500	0,750	60	40
12,000	0,750	60	40
12,001	0,750	60	40
13,000	0,750	95	5

6. Calculations (for each VFA using the external standard method)

6.1. For the calibration curve points

6.1.1. *Determination 1:* Calculate the area of each VFA peak (for each calibration point) in the different calibration standards from the respective chromatograms (Figure 1). To do this, use the equipment's software and determine the area under each peak through integration.

Figure 1. Chromatogram of a 10 mM standard mixture of VFAs. The respective chromatogram also shows adipic acid which can be used as an internal quantification standard. Details of the internal standard method applied to rumen fluid samples can be found in Vargas et al. (2020). Source: Chromeleon™ 7.2 Chromatography Data System (CDS) Software



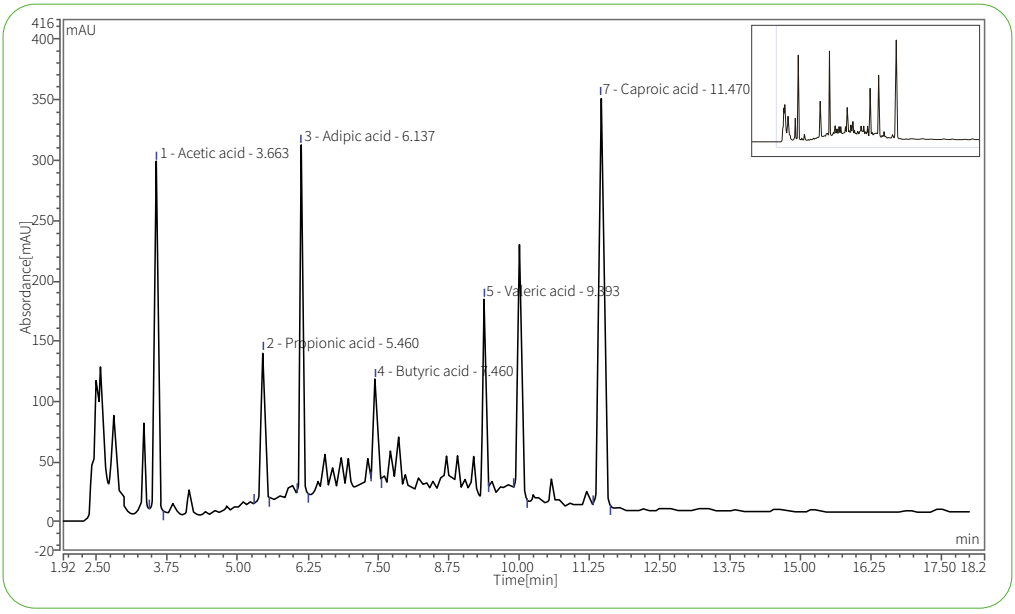
6.1.2. *Determination 2:* Define each compound concentration in the different calibration standards (0, 2, 4, 5, 6, 8 and 10 mM in this protocol).

6.1.3. Create a linear representation of the peak area versus concentration for each compound (calibration curve) and fit the best line using the least squares method. From this process, obtain the equation of the line.

6.2. For samples

6.2.1. *Determination 3:* Identify the VFAs of interest by their respective peak on the chromatogram (Figure 2) and using the standard mixture with known peaks. After this, determine each peak's area by integration using the equipment software.

Figure 2. Bovine rumen fluid sample chromatogram showing the identification of several VFAs. The respective chromatogram also shows adipic acid , which can be used as an internal standard of quantification. Details of the internal standard method applied to rumen fluid samples can be found in Vargas et al. (2020). Source: Chromeleon™ 7.2 Chromatography Data System (CDS) Software



6.2.2. Using determination 3, calculate the VFA concentration using the calibration curve given in section 3.3.1.

6.2.3. The result derived from step 6.2.2 must be multiplied by the appropriate dilution factor (i.e. DF = (4/2) * (1000/500) = 4 in this protocol). This will be the concentration of VFA in rumen fluid in mmol/L or mM.

7. Analysis Time

For 5 samples:

a. Mobile phase and reagents preparation: 10 min

b. Sample preparation: 15 mins.

c. Calibration curve preparation: 100 min (one calibration curve for all analyzed samples).

d. HPLC-DAD configuration and stabilization: 15 mins.

e. Chromatographic analysis run time: 18 min (VFA mix) or 13 min (acetic, propionic and butyric acid only).

f. Data Analysis: 5 mins.

8. Additional Comments

8.1 According to the Hypersil GOLD ™ C18 column manufacturer, the safe buffer use pH range is between 2 and 8. Therefore, if this chromatographic method is optimized in the future using the same column, the use of solvents or mobile phases with a pH outside this range should be avoided, in order to preserve the activity of the stationary phase.

8.2. The column used in the proposed method does not allow the use of 100% aqueous mobile phases (e.g., the use of inorganic acid solutions). Therefore, in case of future optimization of this method, the use of a 100% aqueous mobile phase should be avoided (example use only the buffer as the moving phase).

8.3 Depending on the sample volume of the rumen fluid used, the amounts of reagents required for VFA extraction can be proportionally reduced without significant effects on chromatographic separation.

9. Acknowledgments

Therefore, in case of future optimization of this chromatographic method using the same column, the use of solvents or mobile phases with a pH outside this range should be avoided, in order to preserve the activity of the stationary phase.

The authors thank Conselho Nacional de Pesquisa e Desenvolvimento Científico e Tecnológico (CNPq), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Universidade Federal Rural da Amazônia (UFRA) for the financial support to develop this methodology.

10. References

Berchielli, TT; Pirez, AV; Oliveira, SG. (2011). Nutrição de ruminantes. 2ª edição. Editora Funep, 616 p.

Chromeleon® 7. (2021). Chromatography data System. Thermo Fisher Scientific Inc.

Ferlay, A; Bernard, L; Meynadier, A; Malpuech-Brugère, C. (2017). Production of trans and conjugated fatty acids in dairy ruminants and their putative effects on human health: A review. Biochimie. 141:107-120. doi: 10.1016/j.biochi.2017.08.006.

Insuasti, ASG; Granja-Salcedo, YT; Vargas, JAC; Messana, JD; Sader, APO; Berchielli, TT. (2021). In vitro gas production and fatty acids biohydrogenation of diets containing different unsaturated fatty acids sources plus crude glycerin. Turk. J. Vet. Anim. Sci. 45 (5), Article 18. doi: 10.3906/vet-2101-67

Kozloski, GV. (2012). Bioquímica dos Ruminantes. 3ª edição, Editora UFSM, 212 p.

Valente, ALP; Augustus F; Riedo, CRF. (2019) Quantitative analysis by chromatography. Available from: <www.chemkeys.com>. Accessed: 17 October (2021).

Van Gastelen, S; Antunes-Fernandes, EC; Hettinga, KA; Klop, G; Alferink, SJ; Hendriks, WH; Dijkstra, J. (2015). Enteric methane production, rumen volatile fatty acid concentrations, and milk fatty acid composition in lactating Holstein-Friesian cows fed grass silage- or corn silage-based diets. J Dairy Sci. 98(3): 1915-27. doi: 10.3168/jds.2014-8552.

Van Soest, PJ. (1994). Nutritional Ecology of the Ruminant. 2ª edição. Editora: Comstock; 528 p.

Van Zijderveld, SM; Fonken, B; Dijkstra, J; Gerrits, WJ; Perdok, HB; Fokkink, W; Newbold, JR. (2011). Effects of a combination of feed additives on methane production, diet digestibility, and animal performance in lactating dairy cows. J Dairy Sci. 94(3): 1445-54. doi: 10.3168/jds.2010-3635.

Vargas, JAC; Araújo, TC; Mezzomo, R. (2020). Extraction, identification, and quantification of volatile fatty acids (VFA) in rumen fluid samples using Reverse Phase High-Performance Liquid Chromatography with Diode Array Detector (RP HPLC-DAD). Protocol Exchange. doi: 10.21203/rs.3.pex-1121/v1


20298
93148
93148



PROTOCOL FOR THE EXTRACTION, IDENTIFICATION AND QUANTIFICATION OF VOLATILE FATTY ACIDS (VFAS) IN SILAGE USING REVERSE PHASE HIGH-EFFICIENCY LIQUID CHROMATOGRAPHY COUPLED TO DIODE ARRAY DETECTOR (RP-HPLC-DAD)



Julián Andrés Castillo Vargas
Universidad Nacional Abierta y a Distancia, Medellín, Colombia
Universidade Federal Rural da Amazônia, Parauapebas, Pará, Brasil
Universidade Federal do Norte do Tocantins, Araguaína, Tocantins, Brasil

Tiago Costa de Araújo
Universidade Federal Rural da Amazônia, Parauapebas, Pará, Brasil

Rafael Mezzomo
Universidade Federal Rural da Amazônia, Parauapebas, Pará, Brasil

1. Summary

The short-chain fatty acids (SCFAs) or volatile fatty acids (VFAs) profile in silage provides significant information about its ruminal fermentation pattern, storage conditions and potential utilization by the animal. This chapter describes a methodology for extracting, identifying and quantifying VFAs in silage using reverse phase high- performance liquid chromatography coupled with a diode array detector (RP-HPLC-DAD). The method involves the solid-liquid extraction of VFAs from silage samples. The aqueous silage extract obtained is mixed with 0.85% w/w phosphoric acid and the final extract is analyzed by RP-HPLC-DAD after microfiltration. VFAs are quantified using the external standard method.

2. Introduction

Ruminant production systems around the world are strongly based on forage intake (Reis et al., 2014). Furthermore, it is known that climatic conditions (mainly temperature and rainfall distribution) influence pastures growth and productivity (Lima et al., 2021). Thus, changes in climatic conditions throughout the year determine forages availability with adequate nutritional quality for the production of ruminants. This condition may impair ruminant production, health and reproduction. Therefore, the use of fodder conservation techniques is a common strategy in ruminant production systems to overcome their year-round availability limitation (Coblentz and Akins, 2018).

One of the most common forage preservation techniques uses a combination of anaerobic and acidic environments. This technique is known as silage and the food produced is defined as silage (Silva e Santos, 2016). The silage process aims to preserve the original forages nutritional quality, increasing the forages nutritional availability for different periods of the year. Silages nutritional quality can be assessed using different parameters. Among those parameters, volatile fatty acid profile (VFAs) assessment is an appropriate choice for the quality assessment of a silage because of VFAs profile, the nutritional components of silage and animal productivity are strongly correlated (Wanapat et al., 2015; Lian et al., 2022).

Thus, changes in climatic conditions throughout the year determine the forages availability with adequate nutritional quality for the production of ruminants.

In silages, VFAs profile is composed mainly of lactic, acetic, propionic and butyric acids, whose concentrations may vary depending on the forage source or potential physical or chemical treatments applied

during silage. Thus, this chapter aims to describe a method for extracting, identifying and quantifying VFAs in silage, using reverse-phase high-efficiency liquid chromatography coupled to the diode array detector (RP-HPLC-DAD) as a technique for identification, separation and quantification.

3. Reagents

3.1. Extraction solution

3.1.1. *0.85% (m/m) orthophosphoric acid solution (working solution): carefully* add 1 mL of 85% orthophosphoric acid (Sigma Aldrich®) using a volumetric pipette to a 100 mL volumetric flask. Make up to 100 mL with distilled water.

3.2. Mobile phase components for chromatography analysis

3.2.1. Phase A: 10 mM sodium phosphate buffer solution (pH = 2.6) (1 L): weigh 0.78 g of sodium dihydrogen phosphate dihydrate (Sigma Aldrich®) and mix with 0.34 mL of 85% orthophosphoric acid v/v in a beaker. Add 10 mL of Milli-Q® water to the mixture and transfer it to a 1L Class A volumetric flask. Make up to 1L with Milli-Q® water.

3.2.2. Phase B: Acetonitrile HPLC grade (Sigma Aldrich®)

3.3. Calibration curve preparation for VFA quantification using the external standard method

3.3.1. Calibration curve preparation in the VFA concentration range 2 to 10 mM using a VFA commercial mixture (CRM46975, Sigma Aldrich ®) - corresponding to acetic, propionic, butyric and other VFAs, except lactic acid: Considering that each VFA concentration in the mixture is equal to 10 mM, prepare 6 chromatographic vials and add to each one the quantities of reagent described in Table 1 to prepare the calibration curve in the concentration range mentioned above:

Table 1. Reagents quantities to be used to prepare the VFA calibration curve (acetic acid, propionic acid, butyric acid and other VFAs in the VFA Mix). Source: Authors.

Level	Each VFA final concentration (m)	VFA Commercial Mix Volume (µL)	Milli-Q® water volume (µL)
1	2	200	800
2	4	400	600
3	5	500	500
4	6	600	400
5	8	800	200
6	10	1000	0

Notes:

a. Total volume for each calibration point is 1000 µL.

b. The calibration curves for each fatty acid can also be obtained using individual VFAs. The commercial mix of AGV is suggested for practicality of operation.

3.3.2. *Preparation of the 2 to 10 mM lactic acid calibration curve (Sigma Aldrich®):* Before preparing the calibration curve for lactic acid, it is necessary to prepare a stock solution of 10 mM of lactic acid. To do this, transfer 220 uL of an 85% (w/w) lactic acid solution (solution A) into a 10 mL Class A volumetric flask and make up to 10 mL with Milli-Q® water (solution B). Then transfer 400 µL of solution B into a 10 mL Class A volumetric flask and make up to 10 mL with Milli-Q® water (Solution C). The concentration of lactic acid in solution C shall be 10,039 mM. Considering that the lactic acid concentration is approximately 10 mM, prepare 6 chromatographic pathways and add to each the amounts of Milli-Q® water and Solution C, as described in Table 2.



Table 2. Reagents quantities to be used to prepare the lactic acid calibration curve. Source: Authors.

Level	Lactic acid concentration (mM)	Volume of lactic acid solution C (µL)	Water Volume Milli-Q® (µL)
1	2,01	200	800
2	4,02	400	600
3	5,02	500	500
4	6,02	600	400
5	8,03	800	200
6	10,04	1000	0

4. Equipment

a. HPLC equipped with self-sampler, Hypersil GOLD ™ C18 column, Hypersil GOLD ™ C18 pre-column, oven, and diode array detector.

b. Centrifuge (reaching a minimum of 10,000 x G and 4 °C).

c. Ultra freezer (minimum temperature -20 °C).

d. Analytical balance.

e. Centrifugal tubes of at least 5 mL capacity.

f. Blender.

g. Micropipettes ranging from 100 µL to 5 mL and their respective tips.

h. 100 mL class A Volumetric pipette.

i. Pipette bulb.

j. Syringe and membrane filter (PES membrane), Whatman® brand, 0.45 µm.

k. 5 mL plastic syringes.

l. 1,5-2,0 mL chromatographic vials (amber) with septum and cap.

m. Vacuum filtration equipment.

n. PYREX™ Gooch filter crucibles.

5.Procedure

5.1. VFA Extraction in silage

5.1.1. On the day of analysis, thaw the fresh silage samples at room temperature (20 °C).

5.1.2. Weigh 10 g of fresh silage in an analytical balance and transfer it to the blender vessel.

5.1.3. Add 100 mL of Milli-Q® water (measured using a Class A volumetric pipette to the blender vessel).

5.1.4. Blend the mixture for 30 s at medium speed.

5.1.5. Filter the resulting mixture using a PYREX™ Gooch-type apparatus and vacuum filtration equipment. Retain the filtrate.

5.1.6. Transfer 2 mL of filtrate to 6 mL plastic centrifuge tubes and add 2 mL extraction solution (see section 3.1).

5.1.7. Shake the mixture in a vortex for 30 s at medium speed.

5.1.8. Centrifuge the mixture at 10,000 x g for 15 min at 4 °C.

5.1.9. Recover the liquid phase after centrifugation using a plastic syringe and filter it using the 0.45 µm PES membrane filter. Collect at least 500 µL of the filtrate in a chromatographic vial.

5.2. Chromatographic analysis

5.2.1. Chromatographic analysis of the calibration curve

5.2.1.1. Prepare the calibration curve as described in section 3.3 for reagents.

5.2.1.2. Analyze each point of the curve by RP-HPLC-DAD.

5.2.1.3. dentify and integrate the resulting chromatographic peaks.

5.2.2. Chromatographic analysis of silage samples

5.2.2.1. Place 500 µL of the final extract in a chromatographic vial.

5.2.2.2. Analyze by RP-HPLC-DAD.

5.2.2.3. Identify and integrate the resulting chromatographic peaks.



5.2.3.Chromatographic conditions

a. Column temperature: 30°C.

b. Injection volume: 20 µL.

c. Stationary phase: Hypersil GOLD ™ C18 column (dimensions: 150 x 4.6 mm) equipped with Hypersil GOLD ™ C18 guard column (dimensions: 10 x 4 mm).

d. Working wavelength: 210 nm.

e. Mode: gradient, constant flow: 0.750 mL/min (Table 3).

Table. 3. Gradient used in chromatographic run (lactic acid and VFA Mix). Source: Authors.

Time (min)	Flow (mL/min)	Phase A (Phosphate buffer)	Phase B (Acetonitrile)
Equilíbrio			
-2,000	0,750	95	5
Corrida cromatográfica			
0,000	0,750	95	5
6,500	0,750	60	40
16,500	0,750	60	40
16,501	0,750	60	40
18,000	0,750	95	5

Notes:

a. If only lactic, acetic, propionic, and butyric acids (the most relevant VFAs in silage) need to be separated and quantified, the chromatographic run time may be shorter, and it is suggested to use the program described in table 4.

b. Tables 3 and 4 show a “negative” time. This corresponds to an equilibration time required to perform the chromatographic analysis. In the software of the equipment used, it must be entered this way.



Table. 4. Gradient used in the chromatographic run (lactic, acetic, propionic and butyric acids). Source: Authors.

Time (min)	Flow (mL/min)	Phase A (Phosphate buffer)	Phase B (Acetonitrile)
Equilíbrio			
-2,000	0,750	95	5
Corrida cromatográfica			
0,000	0,750	95	5
6,500	0,750	60	40
12,000	0,750	60	40
12,001	0,750	60	40
13,000	0,750	95	5

6. Calculations (for each VFA using the external standard method)

6.1. For the calibration curve points

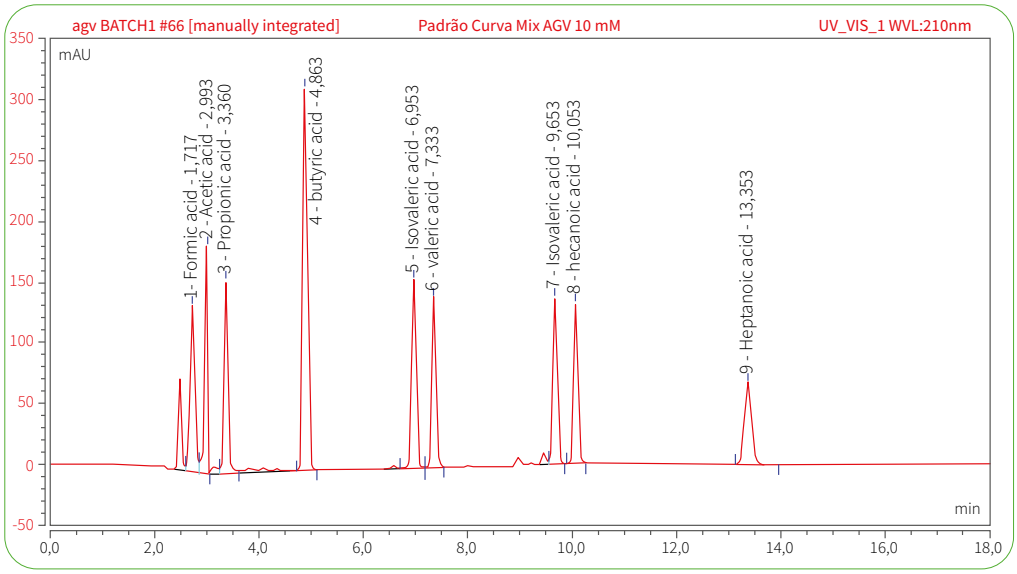
6.1.1. *Determination 1:* calculate the area of each VFA peak (for each calibration point) in the different calibration standards on the respective chromatograms



6.1.2. *Determination 2:* Define each compound concentration in the different calibration standards (0, 2, 4, 5, 6, 8 and 10 mM in this protocol).

6.1.3. Create a linear representation of the peak area versus concentration for each compound (calibration curve), and fit the best line to them using the least squares method. From this process, obtain the equation of the line.

(Figure 1). To do this, use the equipment's software and determine the area under each peak by integration.



For samples

6.2.1. Determination 3: Identify the VFAs of interest by their respective peak on the chromatogram (Figure 2) and using the standard mixture with known peaks and lactic acid standard. After this, determine each peak's area by integration using the equipment software.

6.2.2. Using determination 3, calculate each VFA concentration using the calibration curve given in section 6.1.3.

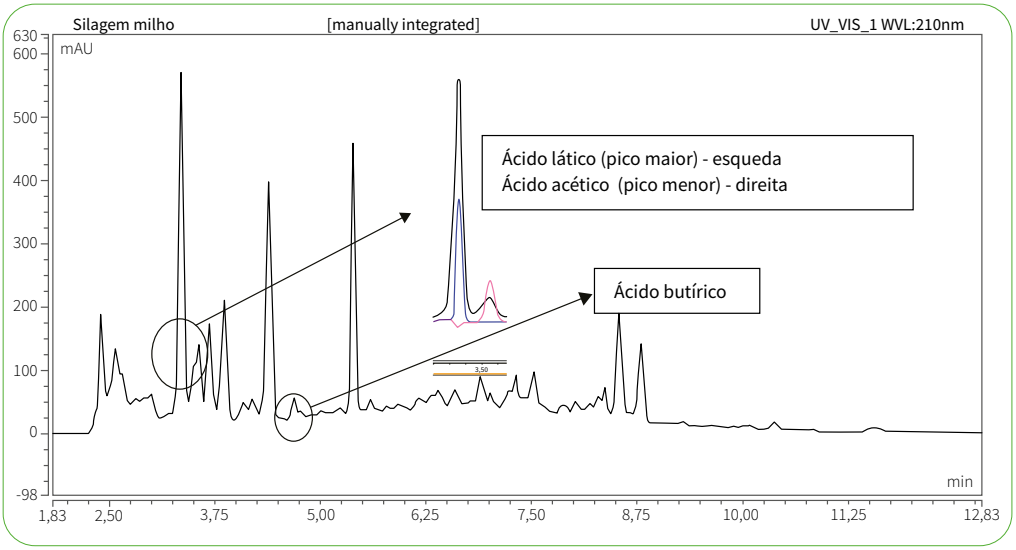
6.2.3. The final concentration of each VFA can be calculated using the following equation:

$$\text{mg AGV/ g MS silagem} = \frac{\text{Concentração AGV (etapa 5)} * 2 * 0{,}1 * \text{Massa molar AGV em análise}}{\text{massa silagem fresca (g)} * \text{MS silagem (\%)}}$$

Where 2 corresponds to the dilution factor used in the method and 0.1 to the volume of Milli-Q® water (100 mL) used in the extraction. The VFA molar mass under analysis must be used independently for each VFA. Lactic acid = 90.08 g/mol, acetic acid = 60.05 g/mol, propionic acid = 74.08 g/mol and butyric acid = 88.11 g/mol. MS of silage, corresponds to the dry matter of silage (%).

The VFA concentration in the silage will be expressed in mg VFA/g MS silage.

Figure 2. Chromatogram of the actual sample of corn silage using the program presented in Table 2. Source: Chromeleon™ 7.2 Chromatography Data System (CDS) Software



7. Analysis Time

a. Mobile phase and reagents preparation: 10 mins.

b. Sample preparation: 30 mins.

c. Calibration curve preparation: 242 min (one calibration curve for all analyzed samples).

d. HPLC-DAD configuration and stabilization: 15 mins.

e. Chromatographic analysis run time: 18 min (VFA mix) or 13 min (lactic, acetic, propionic and butyric acid only). 13 min for lactic acid analysis.

f. Data Analysis: 5 mins.

8. Additional Comments

8.1. According to the Hypersil GOLD TM C18 column manufacturer, the safe buffer use pH range is between 2 and 8. Therefore, if this chromatographic method is optimized in the future using the same column, the use of solvents or mobile phases with a pH

outside this range should be avoided, in order to preserve the activity of the stationary phase.

8.2. The column used in the proposed method does not allow the use of 100% aqueous mobile phases (example: the use of inorganic acid solutions). Therefore, if this method is optimized in the future, the use of a 100% aqueous mobile phase should be avoided (example use only the buffer as mobile phase).

Therefore, if this chromatographic method is optimized in the future using the same column, the use of solvents or mobile phases with a pH outside the range (2 8) should be avoided.

9. Acknowledgments

The authors thank the Conselho Nacional de Pesquisa e Desenvolvimento Científico e Tecnológico t (CNPq), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Universidade Federal Rural da Amazônia (UFRA) for the financial support to develop this methodology.

10. References

Chromeleon® 7. Chromatography data System. Thermo Fisher Scientific Inc.

Coblentz, WK; Akins, MS. (2018). Silage review: Recent advances and future technologies for baled silages. J. Dairy Sci. 101(5): 4075-4092. doi: 10.3168/jds.2017-13708.

Ding, WR; Long, RJ; Guo, XS. (2013). Effects of plant enzyme inactivation or sterilization on lipolysis and proteolysis in alfalfa silage. J. Dairy Sci. 96: 2536 – 2543. doi: 10.3168/jds.2012-6438

Ke, WC; Ding, WR; Xu, DM; Ding, LM; Zhang, P; Li, FD; Guo, XS. (2017). Effects of addition of malic or citric acids on fermentation quality and chemical characteristics of alfalfa silage. J. Dairy Sci. 100(11): 8958-8966. doi: 10.3168/jds.2017-12875.

Lian, T; Zhang, W; Cao, Q; Wang, S; Dong, H; Yin, F. (2022). Improving production of lactic acid and volatile fatty acids from dairy cattle manure and corn straw silage: Effects of mixing ratios and temperature. Bioresour. Technol. 359:127449. doi: 10.1016/j.biortech.2022.127449.

Lima, EM; Vargas, JAC; Gomes, DI; Maciel, RP; Alves, KS; Oliveira, WF; Aguiar, GL; Reis GC; Oliveira, LRS; Mezzomo, R. (2021). Intake, digestibility, and milk yield response in dairy buffaloes fed Panicum maximum cv. Mombasa supplemented with seeds of tropical açai palm. Trop. Anim. Health Prod. 53(1): 178. doi: 10.1007/s11250-021-02626-1.

Reis, RA; Bernardes, TF; Siqueira, GR. (2014). Forragicultura: Ciência, Tecnologia e Gestão dos Recursos Forrageiros. Editora Funep, 714 p.

Silva, T; Santos EM. (2016). Advances in Silage Production and Utilization. Editora Intechopen, 208 p.

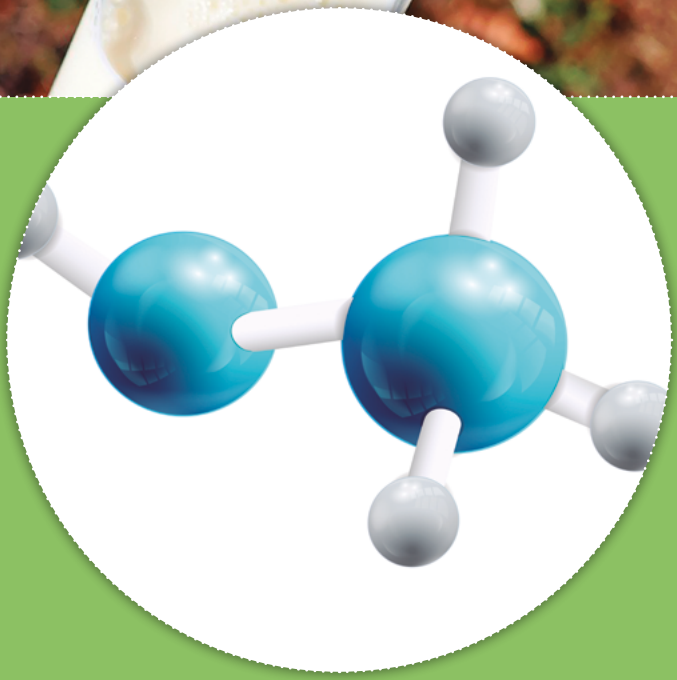
Valente, ALP; Augustus F; Riedo, CRF. (2019) Quantitative analysis by chromatography. Available from: <www.chemkeys.com>. Accessed: 17 October (2021)

Wanapat, M; Cherdthong, A; Phesatcha, K; Kang, S. (2015). Dietary sources and their effects on animal production and environmental sustainability. Anim. Nutr. 1(3): 96-103. doi: 10.1016/j.aninu.2015.07.004.









EXTRACTION, IDENTIFICATION, AND QUANTIFICATION OF BUFFALO MILK PROTEINS USING THE COMBINATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC) AND NEAR INFRARED SPECTROSCOPY (NIRS) MEASUREMENTS



Julián Andrés Castillo Vargas
Universidad Nacional Abierta y a Distancia, Medellín, Colombia
Universidade Federal Rural da Amazônia, Parauapebas, Pará, Brasil
Universidade Federal do Norte do Tocantins, Araguaína, Tocantins, Brasil

Vinícius da Silva Botelho Duarte Gomes
Universidade Federal Rural da Amazônia, Parauapebas, Pará, Brasil
Premix Nutrição Animal, Belém, Pará, Brasil

Tiago Costa de Araújo
Universiadade Federal Rural da Amazônia, Parauapebas, Pará, Brasil

Rafael Mezzomo
Universidade Federal Rural da Amazônia, Parauapebas, Pará, Brasil

Raylon Pereira Maciel
Universidade Federal Rural da Amazônia, Parauapebas, Pará, Brasil

1. Summary

The development of new methods to estimate the number of protein variants of casein (CN) and proteins in buffalo milk whey is necessary to optimize the technological and nutritional properties of dairy products from this species. Thus, this chapter proposes a method to quantify the main casein protein variants and the primary whey proteins in buffalo milk, using the combination of measures derived from reversed-phase high-performance liquid chromatography (RP-HPLC) techniques and near-infrared spectroscopy (NIRS) measurements. The method involves a sequential liquid-liquid extraction of the protein fractions using BisTris buffer solution, guanidine hydrochloride, sodium citrate and dl-dithiotreitol. The final extract obtained must be analyzed by RP-HPLC-DAD, using the total casein and protein value obtained by NIRS for the quantification of the separated and identified proteins.

2. Introduction

Protein variants of casein (CN) and whey proteins in ruminant milk are known for their differential influence on the technological and nutritional properties of milk products (Fox and McSweeney, 2003). Therefore, it is necessary to identify and quantify them efficiently in the milk of different species of ruminants. Among ruminants, buffalo has been the subject of special global attention recently, due to its ability to adapt to different geographical conditions, as well as the wide variety of dairy products that can be manufactured from its milk (Oliveira et al., 2020; Lima et al., 2021). In addition, buffalo milk has shown beneficial effects on human health compared to bovine milk due to its chemical composition (Patiño et al., 2012), which has increased the demands of this product for human consumption.

Therefore, it is necessary to identify and quantify them efficiently in the milk of different species of ruminants. Among ruminants, buffalo has been the subject of special worldwide attention...

The above-mentioned facts have stimulated the development of accurate methods for the determination and characterization of buffalo milk protein, in which currently Reversed-Phase Liquid Chromatography coupled to Mass Spectrometry (LC-MS; Feligini et al., 2009) and High-Efficiency Liquid Chromatography coupled to the Diode Arrangement Detector (RP-HPLC-DAD; Bonfatti et al., 2013) have shown satisfactory results. However,

the mass spectrometry detector is not available in most analytical laboratories due to its high cost. In addition, quantifying proteins using only RP-HPLC-DAD requires pure standards, which are not commercially available for buffalo milk. (Bonfatti et al., 2013). Thus, for quantification using calibration curves, these standards need to be isolated and purified from buffalo milk through preparative liquid chromatography (Bonfatti et al., 2013), a complex method that requires specialized steps and equipment not commonly available in analytical laboratories that are not specialized in metabolite extraction and characterization. This suggests that more applicable methods for real-world conditions need to be proposed.

A strategy to overcome the above-mentioned difficulty could be focused on combining chromatographic techniques with several spectroscopic analysis methods, given their selectivity and accuracy. One of these methods may be near-infrared spectroscopy (NIRS), which has been widely used to determine the composition of milk from ruminants (Melfsen et al., 2020). In a typical chemical analysis report of milk using NIRS, the total protein and casein content can be accurately determined (Tsenkova et al., 2000). Thus, this technique can easily be combined with other analytical techniques to enhance the understanding of the protein composition of ruminant milk.

Buffalo milk's total protein consists of approximately 20% whey proteins, primarily α-lactalbumin (α-LA), β-lactoglobulin (β-LG) and 80% caseins, divided into the main subclasses α-(αS1 and αS2-), βand κ-casein (-CN), which are arranged in micelles (Swaisgood, 1982). Considering that RP-HPLC-DAD provides accurate estimates of casein variants and whey protein proportions., just as NIRS accurately determines total protein and casein content, combining both techniques could facilitate quantitative analysis of buffalo milk protein fractions, overcoming the above-mentioned technical limitations on the exclusive use of RP-HPLC-DAD or LC-MS. Therefore, this chapter presents a method to quantify different protein variants of casein and major buffalo milk whey proteins using the combination of RP-HPLC-DAD and NIRS techniques.

3. Reagents

3.1. Protein extraction solutions

3.1.1. *Solution A:* This solution (pH = 7,0) contains BisTris buffer (batch B-9754, purity > 98%, Sigma Aldrich®, St. Louis, MO, USA) at a concentration of 0.1 M guanidine hydrochloride (lot G-4505. Purity > 99%, Sigma Aldrich®) at 6 M concentration, sodium citrate (Sigma Aldrich®) at 5.37 mM concentration and dl-dithiotreitol (lot 43817, purity > 98%, Sigma Aldrich®) at 19.5 mM concentration.

Note: Prepare this solution daily.

Solution B: this solution contains guanidine hydrochloride (lot G-4505. Purity > 99%, Sigma Aldrich®) at a concentration of 4.5 M. The solvent of this solution is a mixture composed of acetonitrile (HPLC grade): Milli-Q® water: trifluoroacetic acid (TFA, Sigma Aldrich®) in the ratio 100:900:1 (v:v:v).

Notes:

a. Handle the TFA with extreme care.

b. Use an extraction booth to prepare this solution.

3.2. Mobile phases for HPLC

3.2.1. *Mobile phase A:* prepare a 0.1% v/v TFA solution in Milli-Q® water.

3.2.2. *Mobile phase B:* prepare a 0.1% v/v TFA solution in acetonitrile (HPLC grade).

4. Equipment

4.2. Laboratory equipment

a. HPLC equipped with C8 guard column, C8 column, oven, automatic sampler with liquid cooler (minimum: 4 °C) and diode array detector (DAD).

b. Centrifuge (reaching at least 14,000 x g).

c. Freezer (minimum temperature -20 °C).

d. Vortex.

e. Analytical balance.

4.2. Materials

a. 2.2 mL Eppendorf® Safe-Lock Tubes (two per sample).

b. Eppendorf® tubes rack.

c. Volumetric flasks.

d. Automatic micropipettes and tips.

e. Chromatography vials, lids and septum (one set per sample).

f. Micro spatula.

5.Procedure

5.1. Extraction of protein variants

5.1.1. On the day of analysis, if samples are frozen, thaw at room temperature (25 °C).

5.1.2. Take a 1 mL aliquot of milk and transfer it to a 2.2 mL Eppendorf® tube using an automatic pipette.

5.1.3. Add 1 mL of solution A to the 2.2 mL Eppendorf® tube containing the milk sample (1:1 sample: solution A).

5.1.4. Shake the mixture in a vortex for 10 s at medium speed.

5.1.5. Incubate the mixture at room temperature (25 °C) for one hour.

Note:

The room in which the incubation is being carried out can be air conditioned (25º C) using an air conditioning unit. Standardization of incubation conditions contributes to decreasing variations over different processing days.

5.1.6. Centrifuge at 14,000 x g for 5 min.

5.1.7. A top high layer is formed after centrifugation. Then, carefully remove it using a micro spatula.

5.1.8. Take 450 µL of the supernatant and transfer it to a new 2.2 mL Eppendorf® tube. To this same tube, add 1350 µL of solution B (1:3 supernatant: solution B).

5.1.9. Transfer 1 mL of the mixture to a chromatographic vial for HPLC analysis.

5.1.10. Analyze samples by RP-HPLC-DAD. Note: store samples under refrigeration at 4 °C until analysis.

5.2. Chromatographic analysis (conditions)

a. Column temperature: 45 °C.

b. Injection volume: 5 µL.

c. Stationary phase: Hypersil GOLD ™ C8 column (3 µm, 150 x 4.6 mm) equipped with Hypersil GOLD ™ C8 guard column (5 µm, 10 mm).

d. Working wavelength: 214 nm.

Mode: gradient, constant flow: 0,500 mL/min (see elution gradient in table 1).

Table 1. Elution gradient to be used in the chromatographic run. Source: Authors.

Level	Time (min)	Flow (mL/min)	%A	%B	Curvature
1	-13,000		Equilibration		
2	-13,000	0,500	67	33	5
3	-12,001	0,500	67	33	5
4	-12,000	0,750	67	33	5
5	-11,001	0,750	67	33	5
6	-11,000	1,000	67	33	5
7	-10,001	1,000	67	33	5
8	-10,000	1,250	67	33	5
9	-9,001	1,250	67	33	5
10	-9,000	1,500	67	33	5
11	-8,001	1,500	67	33	5
12	-8,000	1,750	67	33	5
13	-7,001	1,750	67	33	5
14	-7,000	2,000	67	33	5
15	-3,001	2,000	67	33	5
16	-3,000	1,500	67	33	5
17	-2,001	1,500	67	33	5
18	-2,000	1,000	67	33	5
19	-1,001	1,000	67	33	5
20	-1,000	0,500	67	33	5
21	0,000	0,500	67	33	5

Level	Time (min)	Flow (mL/min)	%A	%B	Curvature
22	0,000		Chromatographic run		
23	0,000	0,500	67	33	5
24	5,000	0,500	65	35	5
25	5,001	0,500	65	35	5
26	9,000	0,500	63	37	5
27	9,001	0,500	63	37	5
28	18,000	0,500	60	40	5
29	18,001	0,500	60	40	5
30	22,000	0,500	59	41	5
31	22,001	0,500	59	41	5
32	27,500	0,500	59	41	5
33	27,501	0,500	59	41	5
34	28,000	0,500	57	43	5
35	28,001	0,500	57	43	5
36	36,000	0,500	55	45	5
37	36,001	0,500	55	45	5
38	40,000	0,500	55	45	5
39	40,000		End of chromatographic run		

Notes:

a. A = 0.1 % TFA in water; B = 0.1 % TFA in acetonitrile;

b. Negative times correspond to a stationary phase equilibration process, which must be done before each sample injection. During this time, the sample is not injected into the equipment, however, the sample should already be in the chromatograph rack.

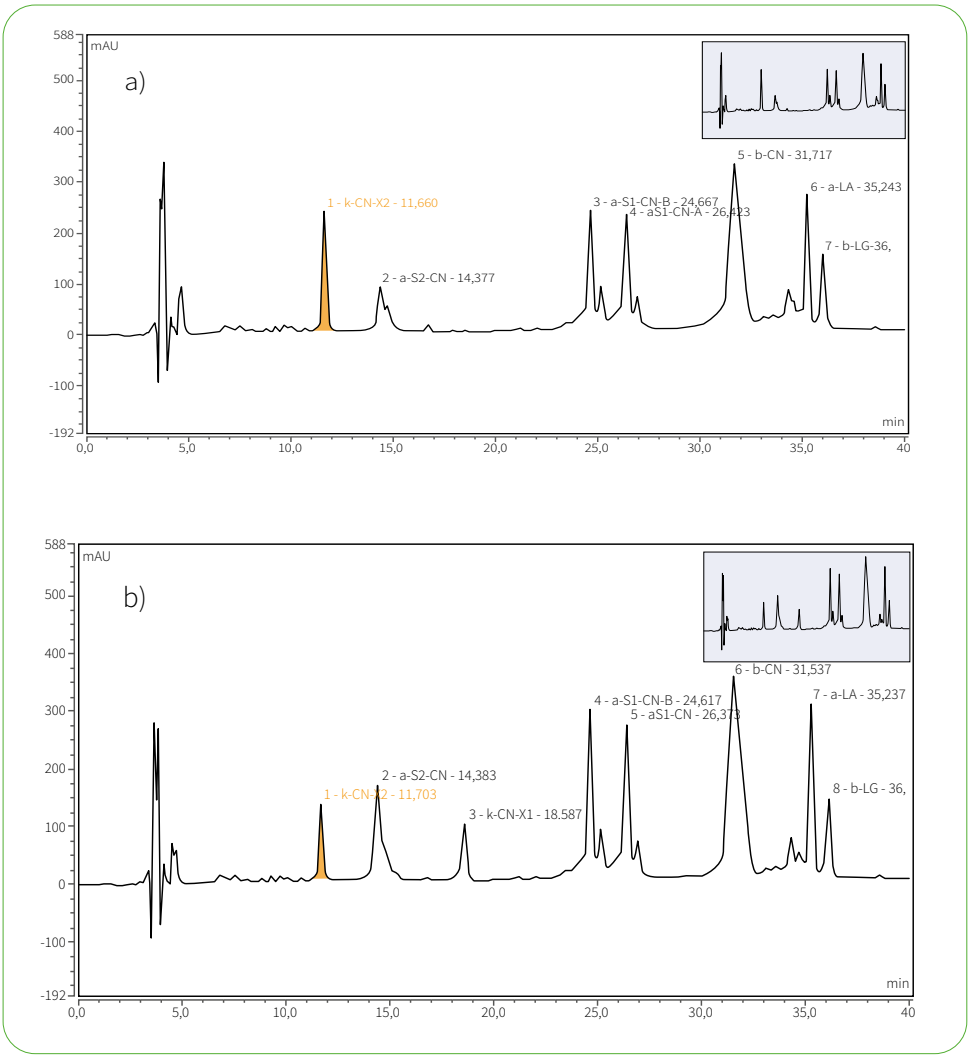
5.3 NIRS analysis

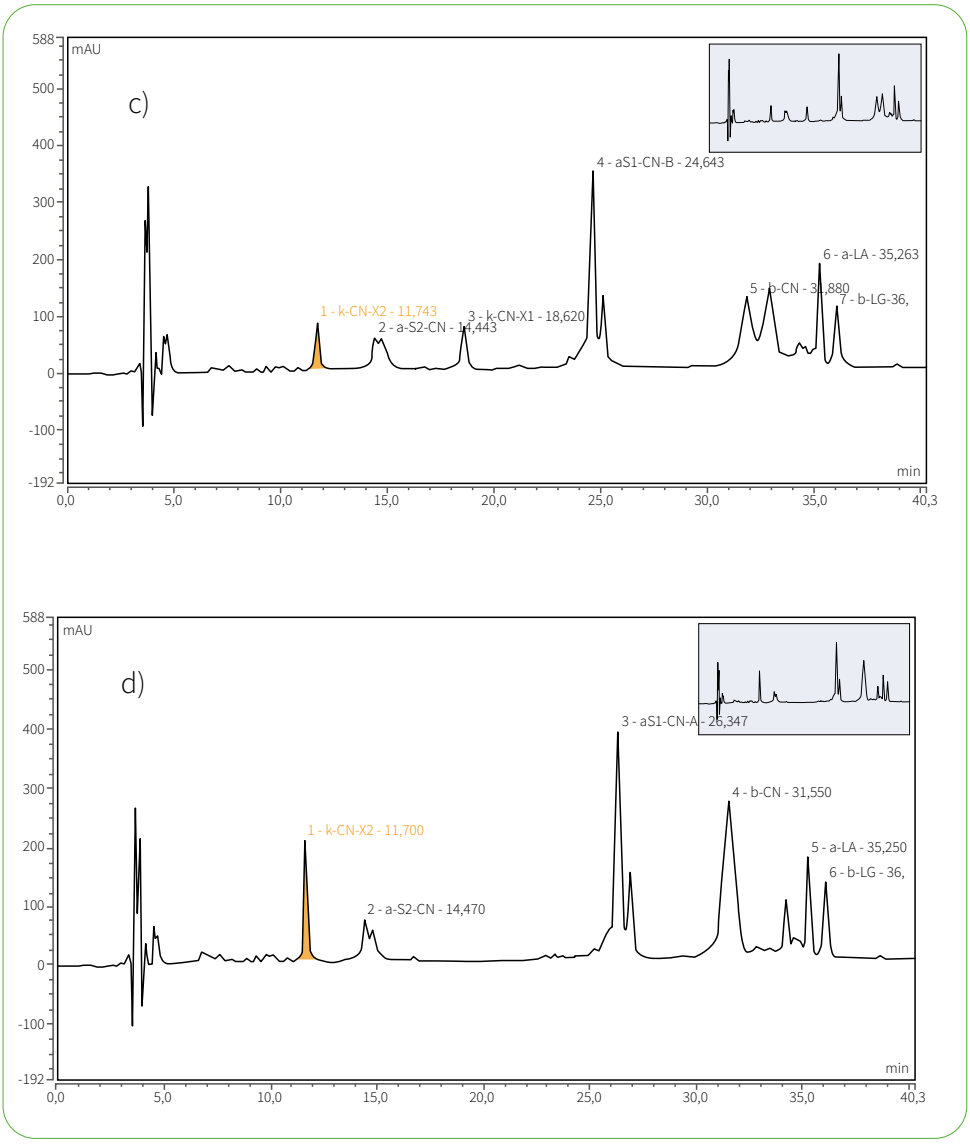
Determine the total protein and casein contents in g/100 g of milk, as well as their density using an NIRS equipment (there are several trademarks for which different analysis protocols are defined). The descriptions of these protocols are beyond the scope of this chapter. For more details on the use of NIRS in animal production sciences, see Evangelista et al. (2022); Giannuzzi et al. (2022) and Yakubu et al. (2022).

6. Calculations

Determine the peak area for each protein in the chromatogram, including variants of caseins(κ-CN X2, α_{s2}-CN, κ-CN X1, α_{s1}-CN B, α_{s1}-CN A e βγ-CN) and proteins in whey (α-La e β-Lg) (Figure 1). Take into consideration that some variants of casein may be absent in the sample.

Figure 1. Chromatographic profiles of buffalo milk from different buffaloes (a, b, c and d). Consider that not all variants of casein are expressed along the milk of different animals.
Source: Chromeleon™ 7.2 Chromatography Data System (CDS) Software





6.2. For calculating the amounts of casein variants κ-CN X2, α_{s2}-CN, κ-CN X1, α_{s1}-CN B, α_{s1}-CN A e βγ-CN), use the following procedure:

6.2.1. Calculate the total peak area of casein variants (sum of peak areas).

6.2.2. Calculate the proportion of each variant of casein according to the following equation:

$$\text{Casein variant ratio} = \frac{\text{peak area of casein variant}}{\text{sum of peak areas of casein variants}}$$

6.2.3. Calculate the concentration of each variant of casein in g/100 g of milk, using the following equation:

Casein variant concentration (g/100g milk) = Casein content (g/100g milk)
x Casein variant proportion

6.2.4. Optional: Casein variant concentration in g/L of milk may be calculated using the following equation:

Casein variant concentrtion (g/L milk) = (Casein variant concentration
(g/100 g milk)) x (milk density (g/mL)) x 10

6.3. For the calculating the amounts of whey protein variants (α-La e β-Lg), use the following procedure:

6.3.1. Calculate the total area of whey protein variants (sum of peak areas).

6.3.2. Calculate the proportion of each whey protein variant according to the following equation:

$$\text{Whey protein variant ratio} = \frac{\text{Peak area of whey protein variant}}{\text{Sum of whey protein peak areas}}$$

6.3.3. Calculate the total whey protein assuming the total whey protein

= casein + whey protein, using the following equation:

Whey protein content (g/100 g milk) = total milk protein (g/100 g milk)
- casein (g/100 g milk).

6.3.4. Calculate the concentration of each whey protein variant in g/100 g of milk according to the following equation:

Whey protein variant concentration (g/100 g of milk) = Whey protein content (g/100 g of milk)
x Whey protein variant proportion

6.3.5. Optional: The concentration of whey protein variants in g/L of milk can be calculated using the following equation:

Whey protein variant concentration (g/L milk) = (whey protein variant concentration (g/100 g milk)) x (milk density (g/mL)) x 10

7. Analysis Time

a. Preparation of solutions and materials: 30 min

b. Protein variants extraction: one batch of 24 samples: 101 min.

c. Chromatographic analysis total time (per sample): 53 min (13 min equilibration + 40 min chromatographic run).

d. NIRS analysis: 5 min.

8. Additional Comments

8.1.Take special care with homogeneous sampling. In some cases, when milk has been stored for long periods or the milk is thawed, it tends to form solid particulate matter. If particulate matter forms, shake the milk container vigorously to collect a representative sample. Otherwise, discard the sample.

8.2. The equilibration program before each sample run is strictly necessary to achieve a successful separation of milk proteins.

8.3. Check the maximum flow limits of your column before using it. Consider that the program involves a maximum flow of 2.0 mL/min, which may be higher than the maximum limit of a different column proposed in this protocol.

Therefore, it is necessary to identify and quantify them efficiently in the milk of different species of ruminants. Among ruminants, buffalo has been the subject of special worldwide attention...

8.4. If the HPLC equipment does not have a refrigerated autosampler module, a conventional refrigerator can be used, and the samples can be injected manually. Processed samples should be stored at 4 °C.

10. Acknowledgments

The authors thank the Conselho Nacional de Pesquisa e Desenvolvimento Científico e Tecnológico t (CNPq), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Universidade Federal Rural da Amazônia (UFRA) for the financial support to develop this methodology.

11. References

Bonfatti, V; Giantin, M; Rostellato, R; Dacasto, M; Carnier, P. (2013). Separation and quantification of water buffalo milk protein fractions and genetic variants by RP-HPLC. Food Chem. 136(2): 364-7. doi: 10.1016/j.foodchem.2012.09.002.

Bonfatti, V; Grigoletto, L; Cecchinato, A; Gallo, L; Carnier, P. (2008). Validation of a new reversed-phase high-performance liquid chromatography method for separation and quantification of bovine milk protein genetic variants. J. Chromatogr A. 1195(1-2):101-6. doi: 10.1016/j.chroma.2008.04.075.

Evangelista, C; Basiricò, L; Bernabucci, U. (2021). An Overview on the Use of Near Infrared Spectroscopy (NIRS) on Farms for the Management of Dairy Cows. Agriculture. 11(4):296. doi: 10.3390/agriculture11040296

Feligini, M; Bonizzi, I; Buffoni, JN; Cosenza, G; Ramunno, L. (2009). Identification and quantification of alphaS1, alphaS2, beta, and kappa-caseins in water buffalo milk by reverse phase-high performance liquid chromatography and mass spectrometry. J. Agric. Food Chem. 57(7): 2988-92. doi: 10.1021/jf803653v.

Fox, PF; McSweeney, PLH. (2003). Advanced Dairy Chemistry. Vol. 1: Proteins. Editora: Kluwer Academic, New York, NY.

Giannuzzi, D; Mota, LFM; Pegolo, S; Gallo, L; Schiavon, S; Tagliapietra, F; Katz, G; Fainboym, D; Minuti, A; Trevisi, E; Cecchinato, A. (2022). In-line near-infrared analysis of milk coupled with machine learning methods for the daily prediction of blood metabolic profile in dairy cattle. Sci Rep. 12(1):8058. doi: 10.1038/s41598-022-11799-0.

Lima, EM; Vargas, JAC; Gomes, DI; Maciel, RP; Alves, KS; Oliveira, WF; Aguiar, GL; Reis, GC; Oliveira, LRS; Mezzomo, R. (2021). Intake, digestibility, and milk yield response in dairy buffaloes fed Panicum maximum cv. Mombasa supplemented with seeds of tropical açai palm. Trop. Anim. Health Prod. 53(1): 178. doi: 10.1007/s11250-021-02626-1.

Melfsen, A; Hartung, E; Haeussermann, A. (2012). Accuracy of in-line milk composition analysis with diffuse reflectance near-infrared spectroscopy. J. Dairy Sci. 95(11): 6465-76. doi: 10.3168/jds.2012-5388.

Oliveira, WF; Lima, EM; Freitas, DR; Dos Santos, SS; Reis, GC; Gomes, DI; Alves KS; Maciel, RP; Vargas, JAC; Mezzomo, R. (2020). Production, chemical composition, and economic viability of Minas Frescal cheese from buffaloes supplemented with açai seed. Trop. Anim. Health Prod. 52(5): 2379-2385. doi: 10.1007/s11250-019-02178-5.

Patiño, EM; Judis, MA; Negrette, MS; Pochon, DO; Cedres, JF; Rebak, G; Romero, AM; Doval, MM; Crudeli, GA. (2012). Influence of fish oil in the concentration of conjugated linoleic acid and omega 6 and 3 in buffalo milk. Arq. Bras. Med. Vet. Zootec. 64, 427-433. doi: 10.1590/S0102-09352012000200024.

Swaisgood, HE. (1982). Chemistry of milk proteins. In: Developments in dairy chemistry. Vol 1. Ed. P.F. Fox, Editora: London: Applied Science Publishers. pp 1-59.

Tsenkova, R; Atanassova, S; Itoh, K; Ozaki, Y; Toyoda, K. (2000). Near infrared spectroscopy for biomonitoring: cow milk composition measurement in a spectral region from 1,100 to 2,400 nanometers. J. Anim. Sci. 78(3): 515-22. doi: 10.2527/2000.783515x.

Yakubu, HG; Kovacs, Z; Toth, T; Bazar, G. (2022). The recent advances of near-infrared spectroscopy in dairy production-a review. Crit. Rev. Food Sci. Nutr. 62(3): 810-831. doi: 10.1080/10408398.2020.1829540







Chapter 4

PROTOCOL FOR THE STUDY OF RUMINAL BIOHYDROGENATION OF UNSATURATED FATTY ACIDS FROM LIPID SUPPLEMENTS MIXED WITH FORAGE USING AN IN VITRO APPROACH

Julián Andrés Castillo Vargas

Universidad Nacional de Colombia, Bogotá, Colombia

Universidad de Antioquia, Medellín, Antioquia, Colombia



1. Summary

The ruminal biohydrogenation of dietary unsaturated fatty acids has an important effect on the final compositional quality of meat and milk of ruminants. Thus, several methodological approaches have been developed to understand and modulate this biochemical process that occurs in the rumen. Therefore, in this chapter, a protocol for performing an *in vitro* test to understand the biohydrogenation of polyunsaturated fatty acids in the rumen is presented. The protocol allows evaluating the effects of different linoleic acid and alpha-linolenic acid relationships in supplements on the production of fatty acid intermediates derived from the biohydrogenation of these unsaturated fatty acids in the rumen, using an *in vitro* approach. In this protocol, experimental steps are proposed to simulate the laboratory scale, the ruminal fermentation process, and thus the biohydrogenation of unsaturated fatty acids, which are subsequently separated, identified and quantified using gas chromatography coupled to the flame ionization detector (GC-FID).

2. Introduction

Understanding the process of biohydrogenation of unsaturated fatty acids (UFA) in the rumen provides valuable information on the use of lipid supplements by ruminants (Harvatine and Allen, 2006; Vargas et al., 2012; Vargas et al., 2018; Vargas et al., 2019). To this end, several experimental strategies were proposed using *in vivo, in situ and in vitro approaches* (Enjalbert et al., 2003; Baldin et al., 2018; Vargas et al., 2018). Each has advantages and limitations discussed in different reviews (Fievez et al., 2007; Jenkins et al., 2008 and Dewanckele et al., 2020); however, the *in vitro approach* has gained particular attention in recent years because it is cost-effective, fast, does not require specialized operators to conduct the test and provides potentially applicable results under *in vivo* conditions. These characteristics allowed the development of multiple *in vitro tests* exploring the effects of different lipid mixtures on the bihydrogenation kinetics of UFA in the rumen, whose results have been used to design nutritional strategies to improve ruminant production and the quality of derived products (Ferlay et al., 2017).

These characteristics allowed the development of multiple in vitro tests exploring the effects of different lipid mixtures on the kinetics of biohydrogenation...

Tilley and Terry (1963) developed an *in vitro test protocol* that has been widely used to evaluate the digestibility of dry matter and nutrients from different feed and forage for ruminants. This method involves two general steps: the first includes fermentation of the forage or feed in ruminal inoculum under anaerobic conditions to simulate ruminal digestion. The second involves digesting the undegraded content in the first step with a mixture of pepsin and HCl to simulate digestion in the posterior digestive tract. From the above-mentioned steps, the first stage of this process (ruminal fermentation) is commonly used to understand the *in vitro* biohydrogenation *of UFA* with successful results (Ribeiro et al., 2007; Vargas et al., 2012 and Vargas et al., 2018). Thus, the purpose of this chapter is to present a general protocol for performing an *in vitro* test based on the Tilley and Terry method (1963) that involves the separation, identification and quantification of UFA precursors, intermediates and products of ruminal biohydrogenation, using gas chromatography coupled to the flame ionization detector (GC-FID).

3. Reagents

3.1. Carbon dioxide (CO_2; gas cylinder).

3.2. McDougall buffer preparation (McDougall, 1948) (Sigma Aldrich®):

3.2. 1. *On the day before the analysis:* prepare a pre-solution in distilled water including the following salts: $NaHCO_3$ (9.80 g/L), Na_2HPO_4 (2.77 g/L), KCl (0.57 g/L), NaCl (0.47 g/L), and MgSO 448.7H128O (0.12 g/L). Keep the pre-solution in a 1 L volumetric flask under refrigeration.

3.2. 2. *On the day of analysis:* add the pre-solution to a 1 L beaker and then add 0.16 g of $CaCl_2.2H_2O$, dissolving this salt under continuous agitation with gentle heating when necessary. When $CaCl_2.2H_2O$ is dissolved and the solution reaches room temperature, add 1.0 g of urea to the mixture. Dissolve the urea and transfer the total mixture into the 1 L volumetric flask. Complete the pre-solution to 1 L with distilled water. Finally transfer the prepared buffer to a plastic or glass bottle and keep it at 39 °C in a thermostated water bath until the inoculum is prepared.

3.3. *$HgCl_2$ solution (2% w/v; Sigma Aldrich®):* weigh 2 g of $HgCl_2$ and dissolve in 100 mL of distilled water.

3.4. Methanol (Sigma Aldrich®).

3.5. oluene (Sigma Aldrich®).

3.6. Sulfuric acid 99.9% (Sigma Aldrich®).

3.7. Dimethyl sulfoxide (Sigma Aldrich®).

3.8 Hexane (Sigma Aldrich®).

3.9. Dichloromethane (Sigma Aldrich®).

3.10. Nitrogen 5.0.

3.11. Helium 5.0 (for GC-FID fatty acid analysis).

Commercial standard mixture of fatty acids (Nu-Check® Prep, GLC-603).

Internal standard (cis-10-17:1, 19:0 or 23:0 fatty acid; Sigma Aldrich®)

4. Equipment

4.1. Laboratory equipment

a. Thermostatic water bath.

b. Analytical balance.

c. Gasification system for CO_2 (composed of CO_2 gas cylinder connected to plastic pipe and pipette tip).

d. Automatic pipettes for transfer of viscous liquid with capillary tips (SOCOREX®).

e. Micropipettes with tips.

f. Ultra freezer.

g. Magnetic stirrer with heating.

h. Potentiometer.

i. Digital thermometer.

j. Lyophilizer.

k. Gas chromatograph equipped with automatic sampler, flame and column ionization detector.

4.2. Materials

a. 100 mL plastic tubes with rubber stoppers with a hole. The hole is essential for the regulation of the internal gas pressure during *in vitro* incubation.

b. Gauze or straining cloth.

c. Thermal bottle.

d. 1 L volumetric flask.

e. 1L beaker.

f. 1 L glass or plastic bottle.

g. Cylinder, graduated pipette or burette of at least 50 mL.

h. 2 mL Eppendorf® Safe-Lock Bullets

i. Eppendorf® bullet support.

j. Micro spatula.

k. Test tubes rack.

l. Tubes with screw cap 16 x 160 mm.

m. Vials with glass inserts, septa and plastic caps.

5. Procedure

5.1. Activities to be carried out on the previous day of the in vitro test



5.1.1. In 100 mL plastic tubes, carefully weigh 500 mg of dried ground forage (e.g. kikuyu grass) using analytical balance.

5.1.2. Seal the tubes with rubber stoppers with a hole and hold them in a test tube rack (Figure 1).

5.1.3. Prepare the lipid mixtures to be tested (treatments) using viscous liquid automatic transfer pipettes and keep them in glass containers under refrigeration.

Figure 1. Tubes with rubber caps with a hole, inside the thermostatic bath to be used in *vitro* incubations. Note: the whole body of the tube should be submerged in water at 39 °C on the day of analysis. Source: Author.



5.2. Activities to be carried out on the day of the in vitro test

5.2.1. Preheat the McDougall buffer to 39 °C in a thermostatic water bath as indicated in section 3.2.2 of the reagents section.

5.2.2. Add the lipid mixtures to the 100 mL plastic tubes prepared the day before the analysis and containing the forage samples. For the addition of lipids, use the viscous liquid automatic transfer pipettes.

5.2.3. Collection of rumen fluid

From a fistulated ruminant (bovine, ovine or caprine), collect at least 50 mL of rumen liquid by filtering it through three layers of gauze or conventional cloth. Carry out the collection in a thermos pre-heated to 39 °C and transport it to the laboratory as quickly as possible.

Note:

a. For preheating the thermos, fill it with preheated water at 39 °C, and empty it at the time of collecting the rumen fluid.

b. The rumen fluid sample must be composed of rumen fluid from three ruminal regions: anterior and half of the ventral sac and the cranial sac (Zijderveld et al., 2011).

5.2.4. Preparation of the inoculum

In a plastic or glass bottle kept at 39 °C in a thermostatic bath, mix the rumen liquid collected in step 5.2.3 with the McDougall buffer in a 1:4 ratio to constitute the inoculum to be used during *in vitro* incubation. After that, adjust the pH of the inoculum to 6.8 bubbling CO_2. Keep the bottle with the inoculum closed and at 39 °C in a thermostatic bath (Figure 2).

Figure 2. Inoculum prepared for *in vitro* incubations. Note: two hoses were fitted to the inoculum vessel, one to bubble CO_2 and the other to serve the inoculum in the incubation tubes. Source: Author.



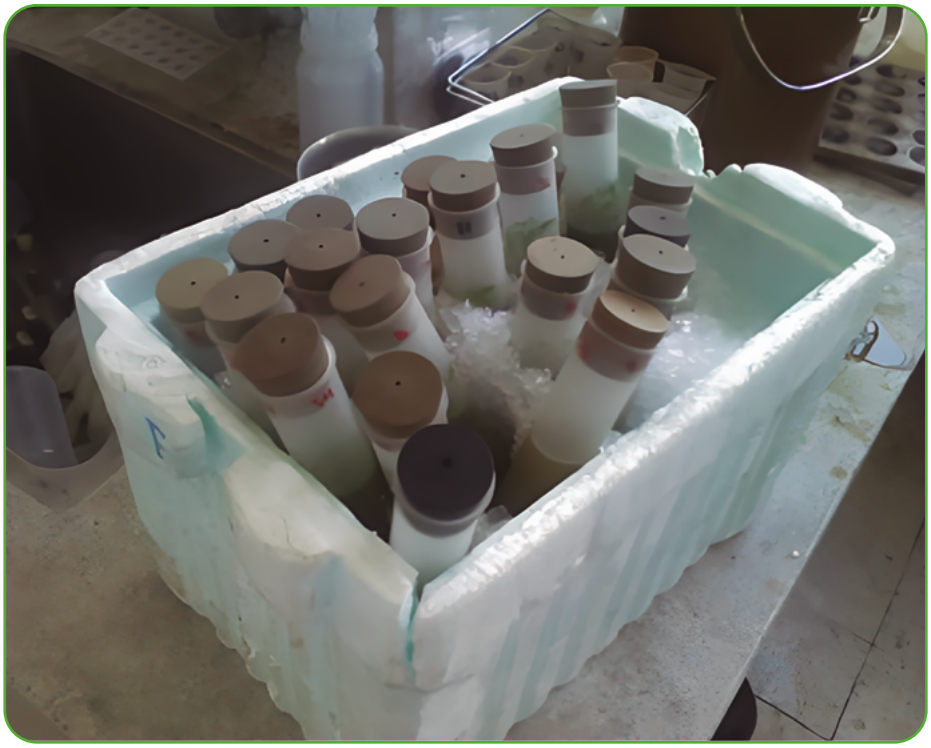
5.2.5. Preparation of incubation systems and *in vitro testing*

5.2.5.1. Place the 100 mL plastic tubes with the mixture of fodder and lipids in the thermostatic bath at 39 °C using the appropriate rack. Ensure that the tubes are balanced at 39 °C by placing them in the thermostatic bath at least 15 min before the in *vitro* test.

5.2.5.2. Take a 100 mL tube from the incubation bath and add 50 mL of pre-heated inoculum at 39 °C using a 50 mL measuring cylinder, pipette or burette, balanced at 39 °C. Gas the tube with CO_2, seal it with the rubber stopper and return it to the thermostatic bath at 39 °C. Repeat this procedure with the other tubes. The tubes should be shaken manually every 2 hours.

5.2.5.3. To evaluate changes in fatty acid concentrations over time (for example, 0, 2, 4, 6, 8, and 16 h), stop UFA biohydrogenation at different times. To do this, remove the plastic tube from the thermostatic bath and add 500 µL of a solution of $HgCl_2$ to 2% m/v and place the tube in an ice bath (Figure 3). Warning: toxic solution, handle with nitrile gloves.

Figure 3. Ice bath for incubation tubes after addition of 2% w/v $HgCl_2$ solution to stop fatty acid biohydrogenation. Source: Author.



5.2.5.4. Transfer the contents of each tube to a 100 mL glass bottle and freeze at -60 °C in ultra freezer.

5.3. Activities to be carried out after the day of the in vitro test

5.3.1. Preparation of incubation systems for fatty acid analysis using GC-FID

5.3.1.1. Place the 100 mL glass vial with the inoculum frozen at -60 °C in a freezer tray, covering the mouth of the bottle with an absorbent paper towel and securing it with an elastic band (Figure 4a). The freeze-drying time is 48 hours and may vary according to the type and number of samples in the freezer. However, the process can only be stopped once all the water has been removed from the bottle (Figure 4b).

Figure 4. Freeze-dried incubation contents. (a) Setup of the vials in the lyophilizer (Note: the vial mouth was covered with an absorbent paper towel and secured with a rubber band). (b) resulting lyophilized incubation system. Source: Author.

a)



b)



5.3.1.2. After freeze-drying, place the vial containing the lyophilized contents in an ultra-freezer at -60 °C until the fatty acid analysis has been performed.

5.3.2. Extraction, methylation and quantification of fatty acids from lyophilized incubation systems (Figure 5; Garcés and Mancha, 1993; Yamasaki et al. 1999):

5.3.2.1. Weigh 50 mg of lyophilized incubation system and an appropriate amount of internal standard (if this method of quantification is to be used) into a 16 x 160 mm screw cap tube.

5.3.2.2. Add the following quantities of reagents to the tube: 2148 µL methanol, 990 µL toluene, 66 µL sulfuric acid 99,9 % w/w, 1000 µL dimethyl sulfoxide (DMSO) and 2 mL hexane.

5.3.2.3. Seal the tube and heat it to 80 ºC for two hours in a water bath.

5.3.2.4. After 2 hours, allow the tube to equilibrate to room temperature and recover the hexane layer (top layer of the mixture) in a 2 mL Eppendorf® Safe-Lock tube.

5.3.2.5. Evaporate the hexane under nitrogen flow and redissolve the resulting residue in 500 µL of dichloromethane.

5.3.2.6. Transfer 250 µL of dichloromethane solution to a chromatographic vial with a suitable glass insert, septum and plastic cap.

5.3.2.7. Inject the mixture into a GC-FID using the following chromatographic conditions:

a. Equipment: Shimadzu GC-2014 (Shimadzu Manufacturing, Inc., Canby, OR, USA) gas chromatograph with automatic sampler.

b. Column: fused silica capillary (Rt-2560, 100 mx 0.25 mm id x 0.2 µm film thickness; Restec®, Inc, Belefonte, PA, USA).

c. Carrier gas: Helium 5.

d. Carrier gas flow: 1.12 mL/min.

e. Chromatographic program:

f. Detector and injector temperatures: 260 and 270 °C respectively.

g. Split mode: 30:1.

h. Injection volume: 1.0 µL.

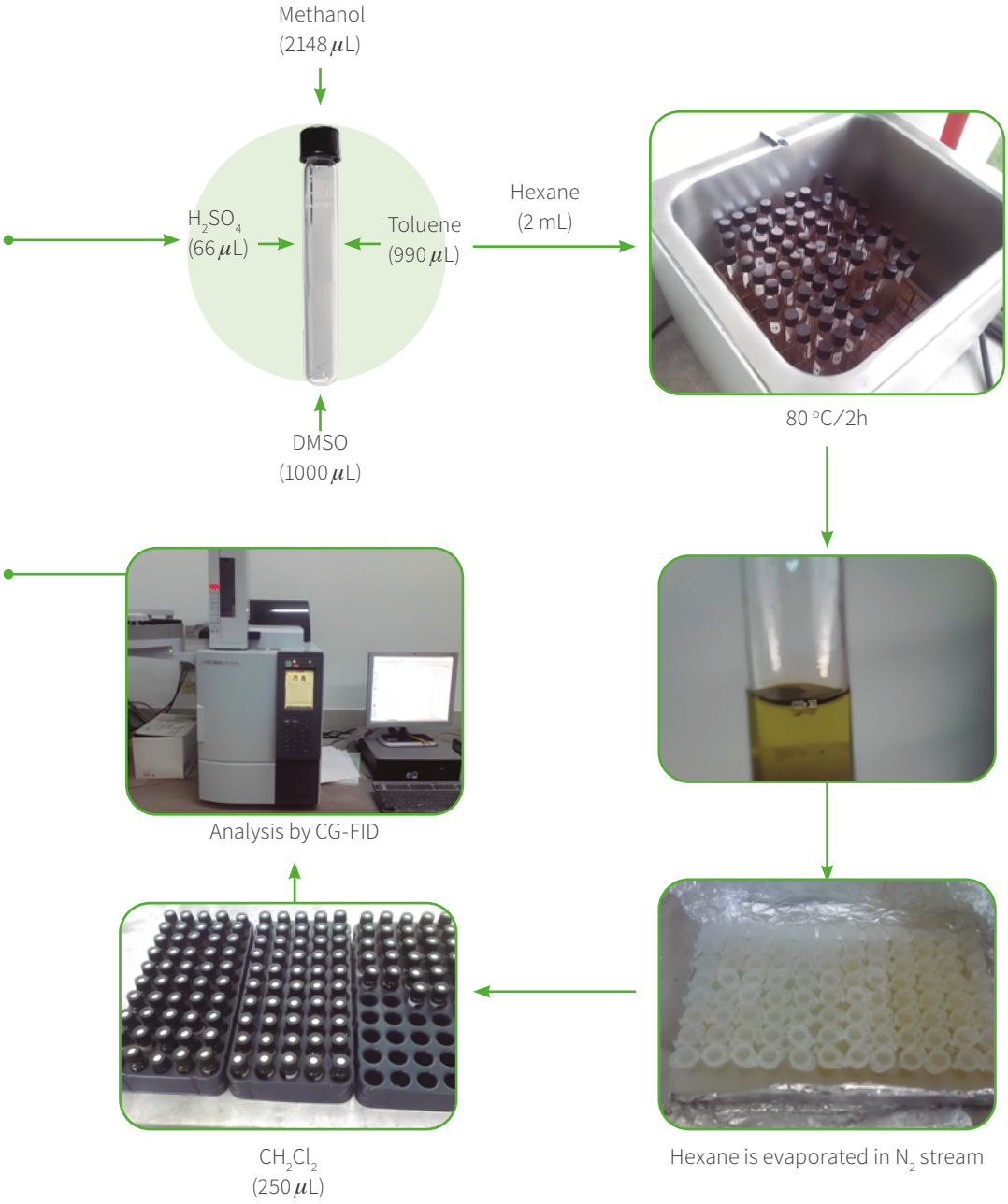
i. Temperature program: The oven temperature is initially set to 140 °C and maintained for 5 min, and after this period, it should be increased by 4 °C/min for an additional 5 min, until reaching 220 °C. Then, it should be increased by 2 °C/min for 10 min until reaching a final temperature of 240 °C.

5.3.2.8. *Fatty acid identification:* The fatty acids in the samples can be identified by comparing their retention times with those observed in commercial standards such as Nu-Check® Prep (Elysian, MN, USA; GLC-603).

5.3.2.9. *Fatty acids quantification:* Quantification can be done by directly comparing peak areas to obtain the relative proportions of fatty acids. The fatty acids concentration or absolute amounts can be obtained using appropriate calibration curves prepared from commercial standards (external standard method) or using cis-10-17:1, 19:0 or 23:0 fatty acids as internal standard (internal standard method). For this meth-

od, an appropriate number of fatty acids needs to be added at the start of the fatty acid extraction and methylation procedure (Section 5.3.2.1).

Figure 5. Scheme for extraction and quantification of fatty acids in incubation systems. Source: Author.



5.3.3. Analysis of fatty acid kinetics

Finally, fatty acid concentrations can be calculated as a function of time to define possible changes of precursors, intermediates and products associated with biohydrogenation over time, as well as the treatment effects on these changes. The association between fatty acid concentration and time can be further analyzed under different statistical and mathematical modeling approaches. For a greater understanding and use of these approaches, it is suggested to review the works published by Ribeiro et al. (2007) Moate et al. (2008) Jenkins et al. (2008) and Vargas et al. (2018).

6. Analysis Time

a. Preparation of the material: 40 min.

b. Preparation of solutions and inoculum: 30 min.

c. Preparation of incubation systems: 5 min per incubation tube.

d. Development of incubations: depends on the time points defined in the experiment.

e. Fatty acid extraction and methylation: 50 h per sample (including freeze-drying, extraction and methylation of fatty acids). This time may vary depending on the lyophilizer used and the number of samples simultaneously lyophilized).

f. Chromatographic analysis of fatty acids: 50 min per sample.

7. Additional Comments

Take special care to maintain the temperature at 39 °C in all instruments and inoculum used during in vitro incubations to avoid the potential death of micro-organisms in the inoculum due to thermal shock.

8. Acknowledgments

The author thanks the Divisão de Investigación de Bogotá (DIB) of the Universidad Nacional de Colombia and the Departamento Administrativo de Ciencia, Tecnología e Innovación (Colciencias) for the financing derived from the project ‘Efecto de la suplementación de vacas lactantes con fuentes ricas en ácidos grasos insaturados sobre el perfil de la leche, transcripción de enzimas lipogénicas, actividad SRBP1 y su relación con diferentes componentes del perfil de ácidos grasos en leche’ (code: 1115-452-21319) administered by the Universidad de Antioquia.

9. References

Baldin, M; Rico, DE; Green, MH; Harvatine, KJ. (2018). Technical note: An in vivo method to determine kinetics of unsaturated fatty acid biohydrogenation in the rumen. J. Dairy Sci. 101(5): 4259-4267. doi: 10.3168/jds.2017-13452

Dewanckele, L; Toral, PG; Vlaeminck, B; Fievez, V. (2020). Invited review: Role of rumen biohydrogenation intermediates and rumen microbes in diet-induced milk fat depression: An update. J Dairy Sci. 103(9): 7655-7681. doi: 10.3168/jds.2019-17662

Enjalbert, F; Eynard, P; Nicot, MC; Troegeler-Meynadier, A; Bayourthe, C. (2003). In vitro versus in situ ruminal biohydrogenation of unsaturated fatty acids from a raw or extruded mixture of ground canola seed/canola meal. J. Dairy Sci. 86(1): 351-9. doi: 10.3168/jds.s0022-0302(03)73613-3

Ferlay, A; Bernard, L; Meynadier, A; Malpuech-Brugère, C. (2017). Production of trans and conjugated fatty acids in dairy ruminants and their putative effects on human health: A review. Biochimie. 141: 107-120. doi: 10.1016/j.biochi.2017.08.006

Fievez, V; Vlaeminck, B; Jenkins, T; Enjalbert, F; Doreau, M. (2007). Assessing rumen biohydrogenation and its manipulation in vivo, in vitro and in situ. Eur. J. Lipid Sci. Tech. 109(8): 740-756. doi: 10.1002/ejlt.200700033

Garcés, R; Mancha, M. (1993). One-step lipid extraction and fatty acid methyl esters preparation from fresh plant tissues. Anal. Biochem. 211(1): 139-143. doi: 10.1006/abio.1993.1244

Harvatine, KJ; Allen, MS. (2006). Fat Supplements Affect Fractional Rates of Ruminal Fatty Acid Biohydrogenation and Passage in Dairy Cows. J. Nutr. 136(3): 677–685. doi: 10.1093/jn/136.3.677

Jenkins, TC; Wallace, RJ; Moate, PJ; Mosley, EE. (2008). Board-invited review: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. J. Anim. Sci. 86(2): 397-412. doi: 10.2527/jas.2007-0588

McDougall, EI. (1948). Studies on Ruminant Saliva. 1. The composition and output of sheep's saliva. Biochem. J. 43: 99-109.

Moate, PJ; Boston, RC; Jenkins, TC; Lean, IJ. (2008). Kinetics of ruminal lipolysis of triacylglycerol and biohydrogenation of long-chain fatty acids: new insights from old data. J Dairy Sci. 91(2): 731-42. doi: 10.3168/jds.2007-0398

Ribeiro, CVDM; Eastridge, ML; Firkins, JL; St-Pierre, NR; Palmquist, DL. (2007). Kinetics of fatty acid biohydrogenation in vitro. J. Dairy Sci. 90(3): 1405-16. doi: 10.3168/jds.S0022-0302(07)71626-0

Tilley, JMA; Terry, RA. (1963). A two-stage technique for the in vitro digestion of forage crops. J. Br. Crassl. Soc. 18: 104-111. doi: 10.1111/j.1365-2494.1963.tb00335.x

Vargas, JAC. (2019). Using multivariate factor analysis to characterize the unbranched fatty acid profile in bovine rumen fluid. Rev. Colom. Cienc. Pecua. 32(3): 175-183. doi: 10.17533/udea.rccp.v32n3a02

Vargas, JAC; Olivera-Angel, M; Ribeiro, CVDM; Caicedo, EED. (2018). In vitro rumen biohydrogenation kinetics of mixed linoleic and alfa-linolenic acids. Rev. Colomb. Cienc. Pec. 31(3): 213-222. doi: 10.17533/udea.rccp.v31n3a06

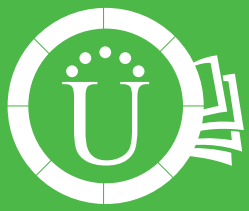
Vargas, JAC; Olivera, M. (2017). Polyunsaturated fatty acid type and ratio in lipid supplements alter the in vitro dry matter digestibility of kikuyu grass (Cenchrus clandestinus). Livest. Res. Rural. Dev. 29: 129. http://www.lrrd.org/lrrd29/7/jcfc29129.html

Vargas, JAC; Olivera, M; Pabón, ML; Carulla, JE. (2012). Reducción de la biohidrogenación del ácido linoleico y alfa linolénico por la adición de diferentes proporciones de ácido eicosapentaenoico y docosahexaenoico. Rev. Colomb. Quim. 41: 395-408.

Yamasaki, M; Kishihara, K; Ikeda, I; Sugano, M; Yamada, K. (1999). A recommended esterification method for gas chromatographic measurement of conjugated linoleic acid. JAOCS. 76: 933-938. doi: 10.1007/s11746-999-0109-0

Van Zijderveld, SM; Fonken, B; Dijkstra, J; Gerrits, WJ; Perdok, HB; Fokkink, W; Newbold, JR. (2011). Effects of a combination of feed additives on methane production, diet digestibility, and animal performance in lactating dairy cows. J. Dairy Sci. 94(3): 1445-54. doi: 10.3168/jds.2010-3635





Sello Editorial
Universidad Nacional
Abierta y a Distancia

Sede Nacional José Celestino Mutis
Calle 14 Sur 14-23
PBX: 344 37 00 - 344 41 20
Bogotá, D.C., Colombia

www.unad.edu.co