Contents

INTRODUCTION 1

1.1 CONTRIBUTIONS 3

1.2 PUBLICATIONS 4

1.3 ORGANIZATION OF THESIS 4

STATE OF THE ART 7

2.1 ORIGIN AND DISTRIBUTION OF JICARO TREE 7

2.2 BOTANICAL PRESENTATION OF JICARO TREE 8

2.3 PHYSICOCHEMICAL COMPOSITION 10

2.3.1 JICARO PULP 10

2.3.2 SEEDS 11

2.4 IMPORTANCE OF JICARO TREE IN THE AGRO-SILVOPASTORAL SYSTEMS IN ARID REGIONS. 12

2.5 JICARO SEEDS OBTAINING PROCESS 15

2.5.1 PROCESSING OF JICARO SEEDS IN TWO LOCALITIES IN THE NORTHWEST OF NICARAGUA 15

2.5.2 STAGES 15

2.5.2.1 Harvesting of the fruits 15

2.5.2.2 Transportation and storage 16

2.5.2.3 Extraction of the pulp 17

2.5.2.4 Flotation 19

2.5.2.5 Drying 19

2.5.2.6 Storage 20

2.5.3 GENERAL VIEW AND COMPARISON 20

2.6 OLEAGINOUS 20

2.6.1 OVERVIEW 20

2.6.2 COMPOSITION 21

2.6.2.1 Storage reserves 22

2.6.2.2 Other components 23

2.6.2.3 Anti-nutritional factors (ANFs) 24

2.6.3 USES OF OLEAGINOUS 25

2.7 ROASTING TECHNOLOGY 28

2.7.1 OVERVIEW 28

2.7.2 METHODS 29
2.7.3 TYPES OF ROASTER 30
2.7.3.1 Batch roaster 30
2.7.3.2 Continuous roaster 31
2.7.3.3 Fluidized-bed roaster 31
2.7.3.4 Microwave roasting 31
2.7.4 PHYSICOCHEMICAL CHANGES INDUCED BY ROASTING 32
2.7.4.1 Physical changes 32
2.7.4.2 Chemical transformations 33
2.8 DEHULLING (DECORTICATION) 35
2.8.1 MECHANICAL DEHULLING 36
2.8.2 CHEMICAL DEHULLING 37

MATERIALS AND METHODS 39

3.1 RAW MATERIAL 39
3.1.1 ORIGIN 39
3.1.2 SAMPLES 39
3.2 PHYSICOCHEMICAL ANALYSIS 40
3.2.1 MOISTURE 40
3.2.2 CRUDE PROTEIN 40
3.2.3 CRUDE FAT 41
3.2.4 ASH 41
3.2.5 FIBER 42
3.2.6 SUGARS 43
3.2.7 MINERAL COMPOSITION 44
3.2.8 PHYTATE DETERMINATION 44
3.2.9 PHENOLIC DETERMINATION 44
3.2.10 TANNINS DETERMINATION 45
3.2.11 TOCOPHEROLS ANALYSIS 45
3.2.12 FATTY ACID COMPOSITION 45
3.2.13 FREE AMINO ACIDS ANALYSIS 46
3.2.14 TRYPSIN INHIBITOR ACTIVITY 46
3.3 MICROSCOPIC ANALYSIS 47
3.3.1 SCANNING ELECTRON MICROSCOPE (SEM) 47
3.3.2 HISTOLOGICAL ANALYSIS OF DEHULLED JICARO SEEDS 47

3.3.3 CONFOCAL LASER SCANNING MICROSCOPY 47
3.4 COLOR MEASUREMENT 48
3.5 ANALYSES OF VOLATILE COMPOUNDS 48
3.5.1 HEADSPACE SOLID PHASE MICRO EXTRACTION (HS-SPME) 48
3.5.2 SOLVENT-ASSISTED FLAVOR EVAPORATION (SAFE) 49
3.5.3 GAS CHROMATOGRAPHY / MASS SPECTROMETRY (GC/MS) OF SAFE EXTRACTS 49
3.5.4 OLFACTORY DETECTION PORT (ODP) OF SPME EXTRACTS 50
3.6 PROTEOMIC ANALYSIS 50
3.6.1 QUANTIFICATION OF PROTEIN 51
3.6.2 REVERSE PHASE HPLC ELECTROPHORESIS 51
3.6.3 ELECTROPHORESIS 51
3.6.4 MASS SPECTROMETRY 51
3.7 ROASTING JICARO SEEDS 52
3.8 DEHULLING JICARO SEEDS 53
3.9 PROCESS DEVELOPED FOR MAKING JICARO MILK 55
3.9.1 RAW MATERIALS 55
3.9.2 HOMOGENIZATION 56
3.9.3 PHYSICOCHEMICAL ANALYSIS OF THE JICARO MILK 56
3.9.3.1 Suspension stability 56
3.9.3.2 Particle size 56
3.9.3.3 Rheological characterization. 57
3.9.3.4 Crude protein 57
3.9.3.5 Crude fat 57
3.9.3.6 Ash 57
3.9.3.7 Color 58
3.9.3.8 Tocopherols 58
3.9.3.9 Volatile compounds 58
3.9.3.10 Analysis of milk microscopy 58
3.10 NUTRITIONAL INTERVENTION STUDY ON THE JICARO MILK 58
3.10.1 DETERMINATION OF THE GLYCEMIC INDEX (GI) 58
3.10.2 METABOLOMICS STUDY OF URINE AFTER CONSUMPTION 59
3.10.2.1 Study design 59
3.10.2.2 Analysis by mass spectrometry 61
3.10.2.3 Data processing 62

RESULTS 65

4.1 PHYSICOCHEMICAL CHARACTERIZATION OF JICARO SEEDS (*CRESCENTIA ALATA* H.B.K.): A NOVEL PROTEIN AND OLEAGINOUS SEED **67**

4.1.1 INTRODUCTION 68

4.1.2 MATERIALS AND METHODS 69

4.1.2.1 Raw materials 69

4.1.2.2 Chemical analysis 69

4.1.2.3 2.3. Soluble sugars 70

4.1.2.4 Fiber content 70

4.1.2.5 Mineral composition 71

4.1.2.6 Phytate determination 71

4.1.2.7 Total phenolic content 72

4.1.2.8 Tannin determination 72

4.1.2.9 Tocopherol analysis 72

4.1.2.10 Fatty acid composition 72

4.1.2.11 Amino acid composition 73

4.1.2.12 Proteomic profiling 73

4.1.2.13 Trypsin inhibitor 75

4.1.3 RESULTS AND DISCUSSION 75

4.1.3.1 Seed structure 75

4.1.3.2 Main chemical composition 75

4.1.3.3 Fatty acid composition 78

4.1.3.4 Amino acid and protein compositions 79

4.1.3.5 Proteomic analysis of jicaro cotyledon 80

4.1.4 CONCLUSIONS 82

4.2 KEY ODOR AND PHYSICOCHEMICAL CHARACTERISTICS OF RAW AND ROASTED JICARO SEEDS (*CRESCENTIA ALATA* K.H.B.) **86**

4.2.1 INTRODUCTION 87

4.2.2 MATERIALS AND METHODS 88

4.2.2.1 Raw materials 88

4.2.2.2 Roasting process 88

4.2.2.3 Jicaro samples for analysis 88

4.2.2.4 Bulk density 89

4.2.2.5 Chemical analyses 89

4.2.2.6 Confocal laser scanning microscopy 89
4.2.2.7 Extraction of volatile compounds 89
4.2.2.8 Analyses of volatile compounds 90
4.2.2.9 Quantification and semi quantification of volatile compounds 91
4.2.2.10 Statistical analysis 92
4.2.3 RESULTS AND DISCUSSION 92
4.2.3.1 Effect of roasting on physicochemical properties of jicaro seeds 92
4.2.3.2 Identification and quantification of volatile compounds 96
4.2.3.3 Determination of key aroma compounds by frequency of detection in olfactometry 98
4.2.4 CONCLUSION 99
4.3 INNOVATIVE PROCESS COMBINING ROASTING AND DEHULLING FOR THE VALORIZATION OF JICARO SEEDS (*CRESCENTIA ALATA* K.H.B) 103
4.3.1 INTRODUCTION 104
4.3.2 MATERIALS AND METHODS 106
4.3.2.1 Raw materials 106
4.3.2.2 Roasting and opening of jicaro seeds 107
4.3.2.3 Physicochemical analysis 107
4.3.2.4 Kinetic modeling 108
4.3.2.5 Mechanical dehulling of the roasted seeds 109
4.3.3 RESULTS AND DISCUSSION 110
4.3.4 CONCLUSION 119
4.4 PRELIMINARY STUDY ON THE ELABORATION OF A VEGETAL MILK FROM DEHULLED JICARO SEEDS 121
4.4.1 INTRODUCTION 121
4.4.2 PROCESS FOR MAKING VEGETAL MILKS 124
4.4.3 RESULTS AND DISCUSSION 127
4.4.3.1 Process flowsheet of jicaro milk 127
4.4.3.2 Effect of soaking temperature and soaking time 128
4.4.3.3 Effect of the presence of the seed coat in jicaro milk color 132
4.4.3.4 Rheological and particle size measurements of jicaro milk 132
4.4.3.5 Physicochemical composition of jicaro milk 135
4.4.3.6 Confocal laser scanning microscopy of jicaro milk 136
4.4.3.7 Suspension stability of jicaro milk 137
4.4.3.8 Clinical study with human volunteers 138
4.4.4 CONCLUSION 143

GENERAL DISCUSSION 145

5.1 PHYSICOCHEMICAL PROPERTIES OF JICARO SEEDS 145

CONCLUSIONS AND PERSPECTIVES 151

6.1 PERSPECTIVES 152

REFERENCES 153

List of Figures

FIGURE 2.1. THE MAYA MYTH OF CREATION ILLUSTRATED BY JOHN JUDE PALENCAR. IT REPRESENTS A JICARO TREE WITH THE OBLONG FRUITS THAT ON THE TRUNK AND BRANCHES _____ 8

FIGURE 2.2. MORPHOLOGY OF THE PARTS OF CRESCENTIA ALATA. A) FRUITS; B) FLOWER; C) LEAF; D) SEEDS _____ 9

FIGURE 2.3. THE COLOR OF THE JICARO PULP BEFORE (LEFT) AND AFTER RIPENING (RIGHT) _____ 10

FIGURE 2.4. TRADITIONAL JICARO POWDER AND BEVERAGE MIXED WITH COW MILK (LEFT) AND WATER (RIGHT) _____ 12

FIGURE 2.5. DISTRIBUTION OF THE TWO MOST COMMON CRESCENTIA SPECIES IN CENTRAL AMERICA: A) CRESCENTIA ALATA AND B) CRESCENTIA CUJETE. _____ 14

FIGURE 2.6. JICARO FRUITS AT THE POINT OF HARVESTING _____ 16

FIGURE 2.7. JICARO FRUITS STORED BEFORE BEING PROCESSED _____ 16

FIGURE 2.8. BLACK JICARO PULP _____ 17

FIGURE 2.9. JICARO SEEDS WASHING _____ 18

FIGURE 2.10. RUPTURE OF THE FRUITS IN THE SEMI-TRADITIONAL PROCESS. (A) FEEDING THE FRUITS IN THE CRUSHER. (B) EXTRACTION OF THE PULP AND SEED. (C) SEPARATION OF THE SHELL _____ 18

FIGURE 2.11. JICARO SEEDS DRYING UNDER THE SUN _____ 19

FIGURE 2.12. A MODEL OF THE OIL BODY SHOWING A TRIACYLGLYCEROL (TAG) MATRIX SURROUNDED BY A MONOLAYER OF PHOSPHOLIPIDS (PL) EMBEDDED WITH THREE CLASSES OF PROTEINS, OLEOSIN, CALEOSIN, AND STEROLEOSIN. _____ 22

FIGURE 2.13. WORLDWIDE OILSEED PRODUCTION IN 2015/2016 (IN MILLION METRIC TONS) _____ 25

FIGURE 2.14. EXPORTS OF OLEAGINOUS AND OLEAGINOUS PRODUCTS BY REGION (OCDE-FAO, 2015) 27

FIGURE 2.15. DIFFERENT STATES OF POPPING OF PADDY USING MICROWAVE ENERGY. A) FULLY POPPED WITH OPEN STRUCTURE; B) POPPED WITHOUT RUPTURE IN SHAPE; C) POPPED WITH IMPROPER EXPANSION AND D) UN-POPPED PADDY _____ 33

FIGURE 2.16. SCHEMA OF THE MAILLARD REACTION. _____ 34

FIGURE 3.1. COFFEE ROASTER USED FOR ROASTING JICARO SEEDS _____ 53

FIGURE 3.2. SATAKE DEHULLER _____ 54

FIGURE 3.3. JICARO SEEDS: RAW (LEFT), ROASTED (MIDDLE) AND DEHULLED (RIGHT) _____ 54

FIGURE 4.1. ANATOMY OF JICARO SEED (CRESCENCIA ALATA) _____ 75

FIGURE 4.2. SEPARATION OF JICARO SEED PROTEINS VIA RP-HPLC (A) FOLLOWED BY 15% SDS-PAGE OF THE RESOLVED PEAKS (B). THE BANDS WITH POSITIVE IDENTIFICATION ARE LABELLED WITH RED CIRCLES IN B. “M” IN FIGURE B IS THE MW MARKER. _____ 81

FIGURE 4.3. CONFOCAL IMAGES OF DEHULLED JICARO SEEDS. OB= OIL BODY; PB= PROTEIN BODY; COB= COALESCENCE OIL BODY; DP= DENATURATION OF PROTEINS, CW= CELL WALL; EV= EXTRACELLULAR VOLUME. (A) RAW AND (B) ROASTED SEEDS SHOWING COALESCENCE OF OIL

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BODIES AND PROTEIN DENATURATION (DP) THROUGH MILD ROASTING. OIL AND PROTEIN BODIES WERE STAINED IN RED AND GREEN, RESPECTIVELY. _____ 92

FIGURE 4.4. REPRESENTATIVE GC-CHROMATOGRAM OF JICARO SEEDS. A) RAW AND B) ROASTED AT 140 °C X 140 S. PEAK NUMBER REFERENCED IN TABLE 4. INTERNAL STANDARD (IS) AND POLLUTION (P) _____ 97

FIGURE 4.5. SCANNING ELECTRON MICROSCOPY (SEM) OF JICARO SEED BEFORE AND AFTER ROASTING. (A) AND (B) SURFACE OF THE SEED COAT BEFORE ROASTING;(C) RUPTURE OF THE SEED COAT AFTER ROASTING; (D) POROUS AND BRITTLE APPEARANCE OF THE SEED COAT AFTER ROASTING _____ 111

FIGURE 4.6. KINETICS AT THREE ROASTING TEMPERATURE (120, 140 AND 160°C) OF A) SEED HEATING, B) SEED MOISTURE LOSS, C) PERCENTAGE OF OPEN SEEDS, AS A FUNCTION OF ROASTING TIME AND D) PERCENTAGE OF OPEN SEEDS, AS A FUNCTION OF SEED MOISTURE CONTENT. ERROR BARS REPRESENT THE STANDARD DEVIATION (N = 3) AND DOTTED-LINES IN B) REPRESENT WATER LOSS MODELING._____ 113

FIGURE 4.7. KINETICS OF (A) LIGHTNESS LOSS; (B) A *VALUE; (C) B* VALUE AS A FUNCTION OF ROASTING TIME AT THREE ROASTING TEMPERATURES (120, 140 AND 160°C). ERROR BARS REPRESENT THE STANDARD DEVIATION (N = 3) AND DOTTED-LINES IN A) REPRESENT LIGHTNESS LOSS MODELING _____ 114

FIGURE 4.8. KINETICS OF A) 2,5-DIMETHYLPYRAZINE; B) ETHYL- 2-METHYLBUTYRATE, AS A FUNCTION OF ROASTING TIME AT THREE ROASTING TEMPERATURES (120, 140 AND 160°C). ERROR BARS REPRESENT THE STANDARD DEVIATION (N = 3). _____ 116

FIGURE 4.9. VARIATIONS IN ROASTING TIME CORRESPONDING TO 90% OPENING SEEDS YIELD (CORRESPONDING TO A RESIDUAL SEED MOISTURE CONTENT OF 2.5%) IN DOTTED LINE AND TO 10% LIGHTNESS LOSS (L* VALUE = 72) IN PLAIN LINE, CALCULATED WITH THE KINETIC DATA OF TABLE 4.9. OPTIMAL ZONE IN GREY._____ 117

FIGURE 4.10. MECHANICAL DEHULLING EFFICIENCY OF ROASTED JICARO SEEDS AS A FUNCTION OF SEED TEMPERING AT VARIOUS MOISTURE CONTENT DURING 2H._____ 118

FIGURE 4.11. FLOWSHEET OF THE OBTAINING OF WHITE JICARO COTYLEDON._____ 119

FIGURE 4.12. FLOWSHEET FOR THE PRODUCTION OF SOYMILK _____ 125

FIGURE 4.13. DESIGN OF THE VALVE OF A CONVENTIONAL HOMOGENIZER (APV GAULIN) _____ 126

FIGURE 4.14. FLOWSHEET OF THE PRODUCTION OF JICARO MILK _____ 127

FIGURE 4.15. JICARO MILK _____ 128

FIGURE 4.16. DRAINING JICARO SEEDS AFTER SOAKING _____ 130

FIGURE 4.17. APPARENT VISCOSITY OF JICARO MILK, SOY MILK AND ALMOND MILK _____ 133

FIGURE 4.18. PARTICLE SIZE DISTRIBUTION FOR DIFFERENT TREATMENTS OF SOAKING_____ 133

FIGURE 4.19. PARTICLE SIZE DISTRIBUTION OF JICARO MILK AFTER HOMOGENIZATION UNDER VARIOUS PRESSURES_____ 134

FIGURE 4.20. COMPARISON OF THE PARTICLE SIZE DISTRIBUTION FOR JICARO, ALMOND AND SOY MILKS _____ 135

FIGURE 4.21. CONFOCAL LASER SCANNING MICROSCOPY OF JICARO MILK (IN GREEN PROTEIN BODY AND IN RED OIL BODIES). _____ 137

FIGURE 4.22. GLYCEMIC RESPONSE AS A FUNCTION OF TIME, AFTER THE INTAKE OF JICARO MILK, SOY MILK AND BREAD_____ 139

FIGURE 4.23. SCORE-PLOT (A), LOADING PLOT (B) AND RESULTS OF PERMUTATION TEST (C) OF THE OSC-PLS-DA MODEL FOR THE COMPARISON OF URINE SAMPLES COLLECTED BETWEEN 0 AND 24 H AFTER CONSUMPTION OF 420 ML OF JICARO MILK (CV-ANOVA=2.10^{-4}, R^2=0.993 Q^2=0.809, R^2 INTERCEPT (0.0, 0.963) AND Q^2 INTERCEPT (0.0, 0.0887) FOR PERMUTATION TEST WITH N=200. _____ 140

FIGURE 4.24. HISTOGRAM OF M/Z 543,139 [M+K] IN URINE SAMPLES COLLECTED AFTER INGESTION OF A CONTROL BEVERAGE AND JICARO MILK._____ 142

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LE NUMERO 1 MONDIAL DU MÉMOIRES

List of Tables

TABLE 2.1. PROXIMATE ANALYSIS OF THE WET AND DRY PULP (%) 10
TABLE 2.2. PHYTOCHEMICAL COMPOSITION OF THE WET AND DRY PULP 11
TABLE 2.3. PROXIMAL CHEMICAL COMPOSITION OF JICARO SEED (G / 100G D.B) 11
TABLE 2.4. PROXIMAL COMPOSITION OF THE ANATOMICAL FRACTIONS OF JICARO FRUIT (G/100G) 11
TABLE 2.5. COMPARISON OF TRADITIONAL AND SEMI-TRADITIONAL PROCESSES OF JICARO SEED 20
TABLE 2.6. NUTRIENT CONTENT OF SOME OILSEEDS (PER 100 G) 21
TABLE 2.7. AVERAGE OIL CONTENT IN SOME OLEAGINOUS (OILSEED AND NUT) 23
TABLE 2.8. ANTI-NUTRIENTS IN SOYBEAN GENOTYPES WITH HIGH PROTEIN CONTENT 25
TABLE 2.9. APPROXIMATE PROPORTIONS OF HULLS AND KERNEL IN SOME OILSEEDS 36
TABLE 3.1. SCHEMA OF URINE SAMPLE COLLECTION 61
TABLE 4.1. MAIN CHEMICAL COMPOSITION OF JICARO SEED 76
TABLE 4.2. FATTY ACID COMPOSITION OF JICARO SEED 79
TABLE 4.3. AMINO ACID COMPOSITION OF JICARO SEED 80
TABLE 4.4. PROTEIN MATCHES OBTAINED FROM PEPTIDE SEQUENCES OF FRACTIONATED JICARO SEEDS VIA MALDI-TOF-TOF MASS SPECTROMETRY 84
TABLE 4.5. CHEMICAL COMPOSITION OF RAW AND ROASTED DEHULLED JICARO SEEDS 93
TABLE 4.6. FREE AMINO ACID COMPOSITION OF DEHULLED JICARO SEED BEFORE AND AFTER ROASTING 95
TABLE 4.7. FATTY ACID COMPOSITION (G/100 G OIL) OF DEHULLED JICARO SEED BEFORE AND AFTER ROASTING 96
TABLE 4.8. IDENTIFICATION AND SEMI-QUANTIFICATION (MG/KG D.B.)[B] ON DB-WAX OF VOLATILE COMPOUNDS EXTRACTED BY SAFE FROM WHOLE RAW SEEDS (RAW), WHOLE ROASTED SEEDS (ROASTED) AND DEHULLED ROASTED SEEDS (DEHULLED ROASTED), AND ODOR COMPOUNDS OF WHOLE ROASTED SEEDS DETECTED BY GC–O ANALYSIS 101
TABLE 4.9. ARRHENIUS PARAMETERS OF MOISTURE AND LIGHTNESS LOSSES OF JICARO SEEDS ROASTED AT THE REFERENCE TEMPERATURE (140°C) 112
TABLE 4.10. COMPOSITION OF VARIOUS RAW OLEAGINOUS USED FOR MAKING VEGETABLE M 122
TABLE 4.11. COMPOSITION OF VARIOUS RAW CEREALS AND PSEUDOCEREALS USED FOR MAKING VEGETABLE MILKS 123
TABLE 4.12. EFFECT OF SOAKING TIME AND TEMPERATURE ON THE YIELD, AND FAT AND PROTEIN CONTENTS OF JICARO MILK 129
TABLE 4.13. EFFECT OF SOAKING TIME AND TEMPERATURE ON COLOR OF JICARO MILK 130
TABLE 4.14. EFFECT OF SOAKING TIME AND TEMPERATURE ON THE CONCENTRATION OF ESTERS AND CARBONYL COMPOUNDS IN MG/KG (WET BASIS) 131

TABLE 4.15. EFFECT OF SOAKING TIME AND TEMPERATURE ON THE CONCENTRATION OF TERPENES AND AROMATIC HYDROCARBONS COMPOUNDS IN MG/KG IN WET WEIGHT BASIS _____ 131
TABLE 4.16. COLOR VALUES FOR DIFFERENT PERCENTAGES OF DEHULLED SEEDS _____ 132
TABLE 4.17. EFFECT OF HOMOGENIZATION ON THE JICARO MILK COLOR _____ 134
TABLE 4.18. PHYSICOCHEMICAL COMPOSITION OF JICARO MILK AND CAKE _____ 136
TABLE 4.19. STABILITY OF JICARO MILK SUSPENSION AFTER 24 H OF STORAGE _____ 138
TABLE 4.20. LIST OF DISCRIMINANT IONS ($P < 0.05$, VIP > 1.5) AROUSED BY THE OSC-PLS-DA MODEL FOR THE ACUTE NUTRITIONAL INTERVENTION WITH JICARO MILK. _____ 142

Chapter 1

Introduction

The calabash tree (*Crescentia alata*), also known as jicaro in Central America, belongs to the bignoniaceae family and is native from Mexico, although it also grows in other tropical and subtropical regions of America. It is a wild tree that adapts very well to the silvopastoral systems of the savannah since it is resistant to droughts and soils poor in nutrients thanks to a root system that spreads depth into the ground.

The jicaro tree is considered to be a multipurpose tree since its different parts are exploited for a variety of usages (as a medicine or food, as a raw material for handicrafts and cooking utensils, as firewood, etc.) (Bass, 2004; Bressani, 1963; Frei et al., 1998). Moreover, its fruits are produced all the yearlong so its exploitation can be done without interruption.

The jicaro fruit consists of a spherical hard-outer shell containing a white pulp with numerous seeds (300-900 seeds per fruit). The pulp is rich in carbohydrates (74%) and proteins (10%) (Solares, 2004) and can be used as a food complement for cattle, incrementing milk productivity between 25 and 50% (Sánchez et al., 2013; Zamora et al., 2001). Jicaro seeds are small, flat and heart-shaped and are surrounded by a dark, coriaceous seed coat, which is strongly adhered to the cotyledons, where the storage reserves are found.

The knowledge about its physico-chemical and nutritional composition is scarce. The first reports about jicaro seeds date from more than half a century ago when Lewy

described its lipidic composition: 31% fat content with its main fatty acids being oleic - (61.8%) and linoleic acids (15%) (Lewy, 1960). Then Bressani demonstrated that jicaro seeds have a good Protein Efficiency Ratio (PER) with no toxic effects for human feed (Bressani, 1963). Since then, there has been very few additional research works about the jicaro seed; the most important one studied its proximal composition and preparation of beverage (Figueroa Madrid and Bressani, 2000). Unfortunately, no information exists about other important biochemical and nutritional properties including its proteomic profile, antinutritional factors, microscopic structure or its volatile compounds. There is no work either about any transformation process or technology applied to jicaro seeds to deliver any added value, even if it has been traditionally employed to prepare a beverage called "horchata", which is part of the traditional diet of Centro American people.

Despite its nutritional properties, the jicaro seed has not been exploited and its commercialization consists almost uniquely in selling it either as the whole seed or as a powder. Taking into account its excellent nutritional value and low cost, the exploitation of the jicaro seed has a huge potential to mitigate the problems of food security in dry regions. This work is comprised within the initiative of research, development and innovation projects in food and nutritional security applied to the region of Central America and promoted by the "Programa Regional de Seguridad Alimentaria y Nutricional para Centroamérica" (PRESANCA II).

This work provides novel and original evidences about the nutritional value and biochemical properties of the jicaro seed and completes the so far partial knowledge about it. This information is exploited in order to apply new processing technologies suitable for the development of nutritional high quality products.

The ultimate goal of this thesis is to propose a manufacture procedure to elaborate a vegetal milk from jicaro seeds that could compete in price and quality with other imported products, such as soymilk. A fundamental step in order to achieve this objective consists of implementing a technology capable of efficiently extracting the cotyledons from the jicaro seeds so as to obtain a material proteinaceous and oleaginous from which the milk would be manufactured.

1.1 Contributions

The contributions of this work are the following:

New knowledge on the physico-chemical and nutritional composition of the seed. In this work, different methodologies of analysis were used for the characterization of the seed that included standardized methods for the determination of the proximal composition, diverse chromatographic analysis for the biochemical composition, a microscopic analysis for the microstructure of the cotyledons as well as a proteomic analysis that allowed to identify the protein families present in the cotyledons. This characterization allowed to have an integral information of the constituents present in the jicaro seed. Novel revelations that can be highlighted include the absence of antinutritional factors in the seeds and the presence of heat shock proteins, which may explain the exceptional resistance of the jicaro to extreme thermal conditions.

The aromatic profile and the key compound of the unroasted and roasted jicaro seeds. For first time Gas Chromatography/Mass Spectrometry (GC/MS) was applied to characterization of volatile compounds of jicaro seeds. The compounds identified belong to the family of aldehydes, esters, alcohols, terpenes, aromatic hydrocarbons typical of other oilseeds. Others volatile compounds are developed in the roasting process (i.e. pyrazines). The combination of olfactometry and gas chromatography was used for the identification of the typical aroma in jicaro seeds. The ethyl-2-metylbutirate was recongnized as the key compound providing this characteristic aroma.

An innovative technology that combines the roasting and tempering of jicaro seeds for the dehulling. The optimization of the roasting allowed to establish the best combination of the parameters of time and temperature to facilitate the mechanical dehulling of the jicaro seed while preserving the white color of the cotyledons and the pleasant aroma of the jicaro. Roasting was optimized for obtaining the best ratio of open seeds, while tempering was used to maximize the final dehulling efficiency by identifying the optimal moisture conditioning prior to the mechanical dehulling procedure.

Valorization and diversification of products based on jicaro. The work provides the guidelines for creating quality and innovative products from the jicaro, including a vegetal milk. The process flowsheet can be applied on a small scale with low cost

equipment. Preliminary results showed that the obtained jicaro milk had a nutritional value higher than soy milk and similar rheological characteristics. Clinical studies completed this analysis, including a glycemic index and a metabolomic study on urine of volunteers after consumption of the jicaro milk.

1.2 Publications

Corrales, C.V., Fliedel, G., Perez, A.M., Servent, A., Prades, A., Dornier, M., Lomonte, B., Vaillant, F. Physico-chemical characterization of jicaro seeds (*Crescentia alata* H.B.K.): a novel protein and oleaginous seed. *Journal of Food Composition and Analysis*.56, 84-92.

Corrales, C. V., Lebrun, M., Vaillant, F., Madec, M. N., Lortal, S., Pérez, A. M., & Fliedel, G. (2017). Key odor and physico-chemical characteristics of raw and roasted jicaro seeds (*Crescentia alata* K.H.B.). Article submitted to Food Research International.

Carla V. Corrales, Nawel Achir, Nelly Forestier, Marc Lebrun, Isabelle Maraval, Manuel Dornier, Ana M. Perez, Fabrice Vaillant, Geneviève Fliedel. Innovative process combining roasting and tempering for the mechanical dehulling of jicaro seeds (*Crescentia alata* K.H.B). Article submitted to Journal of Food Engineering.

Corrales, C.V., Fliedel, G., Perez, A.M., Servent, A., Prades, A., Dornier, M., Lomonte, B., Vaillant, F. A highly nutritious native seed as an innovative food resource in Central America. Food Factor I Barcelona Conference, Barcelona, Spain, 2-4 November 2016.

Corrales, C. V., Lebrun, M., Vaillant, F., Madec, M. N., Lortal, S., Pérez, A. M., & Fliedel, G. (2017). In the heart of volatile compounds of jicaro seeds. Food Factor I Barcelona Conference, Barcelona, Spain, 2-4 November 2016.

1.3 Organization of thesis

The rest of the thesis is organized as follows.

Chapter 2: State of the Art. In this chapter, we review the state of the art. Sections 2.1 to 2.4 give general information about the jicaro tree, including its origin, botanical information, physico-chemical composition of the pulp and seeds and its role in the environment. Section 2.5 describes in detail the obtaining process of the jicaro

seed, presenting both the traditional and semi-traditional approaches. In Section 2.6 general information about the oleaginous, among which the jicaro seeds are included, is described. Section 2.7 describes the roasting treatment and its effects and presents some of the existing roasting technologies. Finally, Section 2.8 explains the importance of dehulling and enumerates the different employed mechanisms.

Chapter 3: Materials and Methods. This chapter describes in detail the samples used throughout this thesis (Section 3.1) and the methods used for the different analysis (Sections from 3.2 to 3.6), the transformation processes (Sections 3.7 to 3.9) and the clinical studies (Section 3.10).

Chapter 4: Results. In this chapter, the results of this thesis are presented in detail. The three first Sections present the results in form of accepted / submitted publications. They include the phyisico-chemical characterization of the jicaro seeds (Section 4.1), the roasting effect on both the volatile compounds and physico-chemical composition of the seeds (Section 4.2) and the combination and optimization of roasting and tempering for the isolation of the jicaro cotyledons (Section 4.3). Finally, Section 4.4 describes the preliminary results on the elaboration of a jicaro milk from the previously isolated cotyledons.

Chapter 5: General Discussion. In this chapter, a general overview of the results of the thesis and the relationships among the different results are described and discussed.

Chapter 6: Conclusions and Perspectives. A general conclusion is given and several guidelines for future research works are proposed.

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Chapter 2

State of the Art

2.1 Origin and distribution of Jicaro tree

The jicaro tree is native to the south of Mexico and spread to Central America, Colombia, Peru and Brazil. However, today, the jicaro tree is widely distributed in the tropics throughout the world. It was cultivated in southern Florida and California (U.S.) and introduced into Bermuda (Humboldt et al., 1963). It is cultivated, as well, in tropical regions of the Asia and Africa (Madhukar et al., 2013).

The jicaro tree is part of the culture of ancient Mesoamerican civilizations. The fruit plays an important role in the *Popol Vuh* (Book of the Community) (Goetz and Morley, 2003), a compilation of myths of the Mayan civilization, which contains a series of stories explaining the origin of the world, of the civilization or of diverse phenomena that occur in the nature, among others. **Figure 2.1** shows the jicaro tree with the fruits attached to the trunk and branches.

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LE NUMERO 1 MONDIAL DU MÉMOIRES

Figure 2.1. *The Maya myth of creation illustrated by John Jude Palencar. It represents a jicaro tree with the oblong fruits that on the trunk and branches*

The jicaro is a multipurpose tree, since each of its elements can be employed for diverse uses. The pulp and seeds can be used either as food for cattle or for human nutritional or as a medicine. The calabash is used as a container for food and drinks (e.g., bowls, cups, spoons), for the manufacture of musical instruments (e.g., maracas) or as a combustible. The trunk is used as wood, mainly for the assembly of barriers to keep the cattle, or as a combustible. Moreover, in Asia, the whole tree is employed for ornamental purposes in gardens.

2.2 Botanical presentation of jicaro tree

Jicaro tree (*Crescentia alata* H.B.K) belongs to the family of the Bignoniaceae. The *Crescentia* is a genus of six species: *Crescentia aculeata* Kunth, *Crescentia alata* Kunth, *Crescentia cujete* L, *Crescentia latifolia* Mill, *Crescentia pinnata* Jacq and *Crescentia portoricensis* Britton (USDA/ARS, 2011).

Common names of the jicaro tree include cuatecomate, ciriani, gua, tecomate, guaje cirián; ayal, comate, cuastecomate, cuate, cuateconate or tecomaxochitl (in Mexico) and guajito, sírial, jícara, morro, zacatecomate or guiro (in the rest of Central America and South America) (Humboldt et al., 1963).

Crescentia alata tree is an evergreen perennial tree and cauliflory, whose flowers and fruits grow directly from the trunk and branches. It has a typical height of 6 – 10 m with pale tan bark, lightly fissured and arching branches.

Leaves are trifoliate, alternate or clustered with large winged petioles (*alata* means “winged”) with a size of 4 – 5 cm long, 0.5 –1.0 cm wide. They are dark green and glossy at the upper surface.

The flowers are hermaphrodite and tubular. They are only open at night, during which they produce large quantities of nectar. Pollen is in the dorsal side of the flower, leading to its eventual deposition on the head and shoulders of pollinating bats (Janzen 1983). Flowers have a musty odor (musk); the calyx is 2 cm long and bipartite with a corolla of 3.5 – 6 cm long and 0.7 – 1.2 cm wide. They are 5-lobed, with lobes being triangular and fused at the base. Lobes are tubular, cup-like and split into two halves till the base. Flowers have a yellowish with purplish color and a bell shape (see **Figure 2.2**) (Madhukar et al., 2013).

Crescentia alata have a unilocular indehiscent fruit of 7 – 11 cm in diameter and rounded shape with a hard, woody shell (pericarp). The fruits grow and ripen slowly, remaining on the tree for 6 to 7 months. Then, they fall to the ground and degrade with time. When they are over-ripe their color changes: their pericarp becomes red-yellowish and their pulp loses moisture becoming black (Niembro Rocas, 2002).

Jicaro's seeds are dicotyledons, small (7 – 7.5 mm long, 5.4 – 7.0 mm wide and 1.0 – 2.0 mm thick), flat, non-winged and embedded in pulp. The seeds are obovate, with an emarginated apex and laterally flattened. The seed coat is dark brown, furrowed with dots, opaque and coriaceous.

The morphology of each of the parts of the jicaro tree is shown in **Figure 2.2**

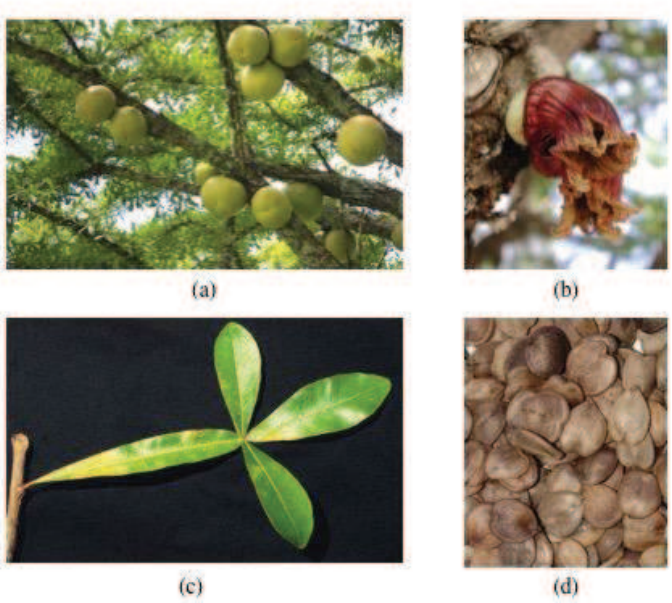

***Figure 2.2**. Morphology of the parts of Crescentia alata. a) fruits; b) flower; c) leaf; d) seeds*

2.3 Physico-chemical composition

2.3.1 Jicaro pulp

All species of the *Crescentia* genus are composed of a fleshy fruit containing a sweet white pulp. When the fruit ripens the pulp becomes black and sticky (Olmstead et al., 2009).

Figure 2.3. *The color of the jicaro pulp before (left) and after ripening (right)*

The pulp is not edible in its natural state due to their phytochemical composition; it contains alkaloids, saponins and cyanogenic glycoside as hydrocyanic acid (HCN) (Ogbuagu, 2008). However, after ripening it can be used as food for cattle and poultry.

The ripen pulp is a good source of carbohydrates and proteins (**Table 2.1**). In its composition, they have been found iridoid glycosides, polyphenols, furanonaphthoquinones with anticancer activity (**Table 2.2**) (Heltzel et al., 1993; Kaneko et al., 1997; Valladares and Rios, 2007).

Pulp and seeds have also been used in traditional pharmacopoeia for its healing properties in respiratory, digestive and inflammatory conditions (Autore et al., 2001; Frei et al., 1998; Morton, 1968; Rojas et al., 2001; Solares, 2004). The pulp has also been used in Nicaragua for the manufacture of a luxury liquor called Jicor (Jochims Karsten, 2004).

Table 2.1. Proximate analysis of the wet and dry pulp (%)

Pulp	Moisture	Ash	Ether extract	Crude protein	Crude fiber	Carbohydrate	Dry matter
Wet	68.68	2.92	4.38	7.67	0.70	15.65	31.32
Dry	12.52	3.74	0.70	10.01	4.88	68.13	87.48

Ogbuagu, (2008)

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Table 2.2. Phytochemical composition of the wet and dry pulp (%)

Pulp	Alkaloids	Flavonoids	Saponins	HCN	Tannins	Phenols
Wet	0.74	0.52	0.70	0.28	0.64	0.46
Dry	0.76	0.38	0.34	0.23	0.85	0.14

Ogbuagu, (2008)

2.3.2 Seeds

Jicaro seeds are released from the ripe fruit after maceration, then dried under the sun to 9%-12% of moisture content. For their consumption, the seeds usually go through a process of roasting. First studies of its nutritional potential date back half a century ago. Lewi (1960) was the first to characterize the oil fatty acid profile and Bressani (1963) evaluated the potential nutritional value of jicaro flour as follows (in mg/g of N_2): Arginine 678, Histidine 181, Isoleucine 284, Leucine 367, Lysine 266, Methionine 158 (includes methionine + cysteine), Phenylalanine 242, Threonine 190, and Valine 310.

Later, Figueroa Madrid and Bressani (2000) characterized the chemical composition of the pulp and seeds. Its most outstanding characteristic is the high content of fat and protein as shows **Table 2.3**.

Table 2.3. Proximal chemical composition of jicaro seed (g / 100g d.b)

	Jicaro seed (sun dry)	Roasted jicaro seed (*)
Moisture	8.47 ± 0.81	5.80 ± 0.37
Fat	34.85 ± 1.58	33.75 ± 1.21
Protein (Nx6.25)	25.69 ± 0.36	27.73 ± 0.43
Ash	4.58 ± 0.22	4.93 ± 0.34

Roasting at 90-110ºC during10 min (Figueroa Madrid and Bressani, 2000)

The jicaro oil presents a good profile of unsaturated fatty acids, mainly oleic (61.8 %) and linoleic acid (15%) making it a species with the great potential to produce a high quality edible oil (Lewy, 1960).

Table 2.4. Proximal composition of the anatomical fractions of jicaro fruit (g / 100g)

	Pulp and seeds	Pulp	Seeds
Moisture	68.71 ± 1.12	71.67 ± 0.69	41.66 ± 0.90
Fat (*)	18.36 ± 0.82	4.55 ± 0.19	37.95 ± 1.43
Protein (N x 6.25)	18.62 ± 0.36	12.94 ± 0.33	26.41 ± 0.48
Ash (*)	6.85 ± 0.91	8.10 ± 0.56	4.56 ± 0.78

(*) Dry matter (Figueroa Madrid and Bressani, 2000)

After extracting the oil, it remains a cake residue with a high content of protein, 64.9% (Bressani, 1963). This is usually employed in animal feeding.

In Central America, the jicaro seeds are sold in the market to be used in the preparation of a traditional beverage called “horchata” or “morro” (see **Figure 2.4**). This beverage presents pleasant sensory properties with a great caloric value. Indeed, it has been part of school nutrition programs in Central America (Gutiérrez, 2012).

Figure 2.4. *Traditional jicaro powder and beverage mixed with cow milk (left) and water (right)*

This leads us to define this plant as a multipurpose tree, as each of its parts is used for a given purpose. From the fruit all the components are employed: the seeds for human consumption, the pulp for medical purposes and animal feeding and the pericarp as the raw material for handicrafts and cooking utensils. On the other hand, the trunk is used for the construction of fences or as firewood and the branches are also used as firewood.

In addition, as we will detail in the next section, the jicaro tree can be well integrated in the agro-silvopastoral systems of dry regions, providing shade to the cattle and allowing the growth of pastures.

2.4 Importance of jicaro tree in the agro-silvopastoral systems in arid regions.

Land usage systems integrating trees and agriculture have been exploited for thousands of years, although it was not until 1977 that the term **agroforestry** was coined for such systems (Bene Beall, W.G., Côté, A., 1977). This system allows that many developing countries integrate forests and trees as part of livestock production systems, helping to support the production system, the economy of rural families

through the production of firewood, timber, live fences, fodder, fruits, protect water sources and provide shade for livestock (Sánchez et al., 2013).

Agroforestry systems have been classified according to their combination of components (Smith, 2010):

- **Silvorable**: trees and crops
- **Silvopastoral**: trees and animals
- **Agro-silvopastoral**: trees with crops and animals. Trees with pastures and animals are also considered agro-silvopastoral systems by some authors (Alao and Shuaibu, 2013).

Jicaro trees are well adapted to agro-silvopastoral systems. They are useful for the protection of livestock in arid areas, as they grow along with pastures that serve as food for livestock when the dry season greatly reduces the herbaceous forage (Bucheli et al., 2013).

The jicaro tree grows in open dry forests and the savannah. It has a preference for the sandy, porous and clay soils; however, it can grow in any soil (FAO, 2000). In Nicaragua, the distribution of the jicaro tree varies from a dense distribution (200 to 300 trees/ha) to widely scattered distributions (1 to 20 trees/ha).

Jicaro trees spread naturally by seeds that take 2 to 3 months to germinate. Recently people have begun to plant them by cutting (Cordero and Boshier, 2003). Flowering occurs throughout the year but especially in March-July. The harvest is concentrated in two periods: from August to October and from December to April. However, the fruit production is distributed throughout the year.

Jicaro trees begin to produce fruits when between 4 and 5 years old with a maximum production when they are 8 to 10 years old with a production between 60 - 200 fruits per tree. The average weight of a fruit is 370 g, so one ton corresponds to have 2740 fruits. About 12 years the production is reduced but they continue to produced fruits all their life (> 50 years) (Carballo et al., 2005).

Two species of the jicaro tree are mostly distributed in the Central America: *Crescentia alata* and *Crescentia cujete* (see **Figure 2.5**); both are similar in morphology and distribution. *Crescentia cujete* is considered to be a domesticate tree which has

simple oblongate leaves and much larger fruits and *Crescentia alata* is a non-domesticate tree (Bass, 2004; Gentry, 1980).

The fruit of both species consists of a hard-outer shell (25% weight). Inside it contains a white pulp (50-60% weight) with numerous seeds. During the ripening of the fruits the pulp changes its color, turning black. At this point the pulp serves as food for livestock, increasing milk production between 25 and 50% (Sánchez et al., 2013; Zamora et al., 2001).

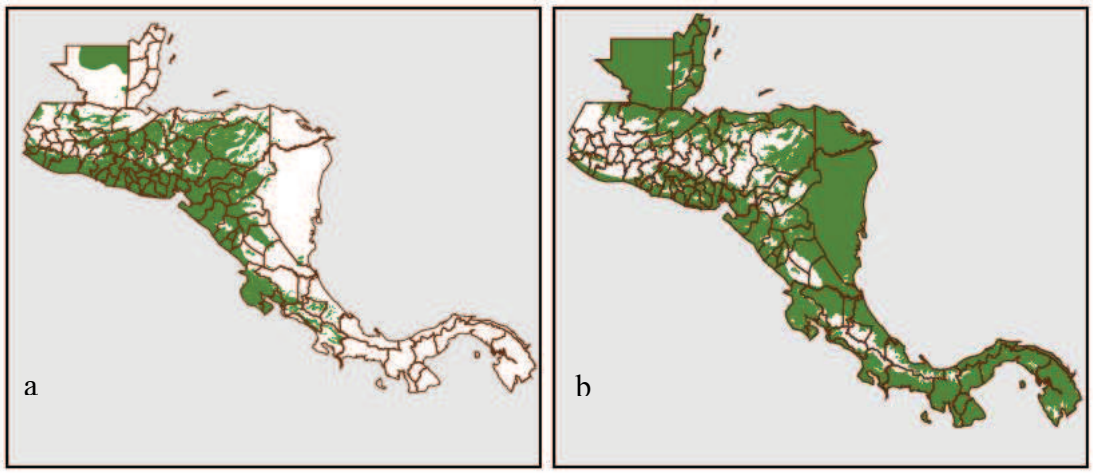

Figure 2.5. *Distribution of the two most common Crescentia species in Central America: a) Crescentia alata and b) Crescentia cujete.*
(Cordero and Boshier, 2003)

Crescentia alata or jicaro "sabanero" is a typical tree of savannah vegetation and grows wild in tropical dry forests of the pacific, central and northern parts of Central America. It can tolerate extensive periods of extreme humidity or drought.

Crescentia cujete is more tolerant to the lack of sun and is more adapted to humidity than *Crescentia alata*. They grow on heavy terrain, like vertisols, are very resistant to poor soils and tolerate flooding.

Studies on the species biodiversity carried out by Arango et. al., (2009) showed that both species could be hybrid forms as a result of the interspecific interaction of the genus Crescentia.

2.5 Jicaro seeds obtaining process

This section focuses on two alternative processes for obtaining jicaro seeds (*Crescentia alata*), here denoted traditional and semi-traditional. The information collected comes from the observation of each one of the selected processes. The traditional process is carried out by small producers, who obtain the seeds with a traditional handcrafted method. The semi-traditional process is not very different from the traditional one, but introduces the usage of machinery and other additional treatments which allow the production of larger volumes of seeds.

2.5.1 Processing of jicaro seeds in two localities in the northwest of Nicaragua

The jicaro "sabanero" is presented in Nicaragua in two types of population: one of them in dense groups (150 – 200 trees per 1 ha), and another very dispersed distribution (1 – 15 trees per 1 ha). The jicaro produces an average of 750 fruits per year, each tree offers 112 kg of edible matter annually, of which 52 kg are seeds and 60 kg is pulp (Carballo et al., 2005).

All the details presented below were obtained from the caserío Los Zarzarles (latitude: 12.66°N, longitude: -86.44°W, altitude: 115.7 m) in the municipality of León, pacific region of Nicaragua. In this region, there are several jicaro plantations and both processes coexist. The semi-traditional process is carried out by a company called Jicaro S.A., owned by Karsten Jochims, and it is used on a single plantation owned by the same company. It has an average yield of 600 tons of fruit per hectare and year for a total production of 3 tons per week. Jicaro seeds coming from the rest of the plantations are handled with the traditional process.

The general procedure is performed in several stages.

2.5.2 Stages

2.5.2.1 Harvesting of the fruits

The fruits are harvested manually when the color of the shell turns yellowish, as shown in **Figure 2.6**. Fruits can fall to the ground or are removed from the trees by cutting them using a wooden stick with a hook at the end.

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Figure 2.6. *Jicaro fruits at the point of harvesting*

2.5.2.2 Transportation and storage

After being harvested, the fruits are transported to the point of process, where they remain for several days under sunlight until dehydration is completed (**Figure 2.7**). In the traditional process, they are kept for a period of 8 days, whereas in the semi-traditional process they only remain 5 days. This stage is probably implemented to enhance natural dehydration of the calabash and permit easier handling by workers.

Figure 2.7. *Jicaro fruits stored before being processed*

2.5.2.3 Extraction of the pulp

Since the peel of the fruit is very hard, a hammer is used to break the fruit and extract the pulp. In the traditional process, the fruits are crushed using pieces of wood and stones. The ripen fruits present a black pulp Figure **2.8**.

Phytochemical studies of fresh fruit pulp report the presence of crescentic acid, tartaric acid, citric acid and tannic acids, two acid resins (soluble in alcohol and another in ether) (Carballo et al., 2005; Ejelonu, 2011; Kaneko et al., 1997)

The black pulp of the ripen fruits has been employed since the eighteenth century to prepare a tonic used to relieve different respiratory infections such as cough, asthma, bronchitis, tuberculosis, and breast pain (Agarwal and Chauhan, 2015; Argueta A. et al., 1994; Rojas et al., 2001).

The pulp and seeds of the fruit also serve as food for cattle, horses, pigs and chickens (Botero and De La Ossa V, 2011; INTA, 1996; Kyvsgaar and Urbina, 1996; Sánchez et al., 2013) .

Figure 2.8. *Black jicaro pulp*

The pulp (which contains the seeds) is placed in a container, where it is added cool water and washed vigorously. The seeds are removed by hand-squeezing the pulp while washing it with the water (see Figure **2.9**). This process facilitates the release of the seeds, which are collected in a strainer. In this step a lot of foam is produced, which could be associated with the presence of saponins (Ejelonu, 2011; Nwosu, 2008).

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Figure 2.9. *Jicaro seeds washing*

In the semi-traditional process, the fruits are placed inside a machine with blades which grinds the calabash using a rotation mechanism. The machine is fed with water at room temperature that helps the pushing of the fruits. The pulp and seeds are separated from de shell as showed in the **Figure 2.10**

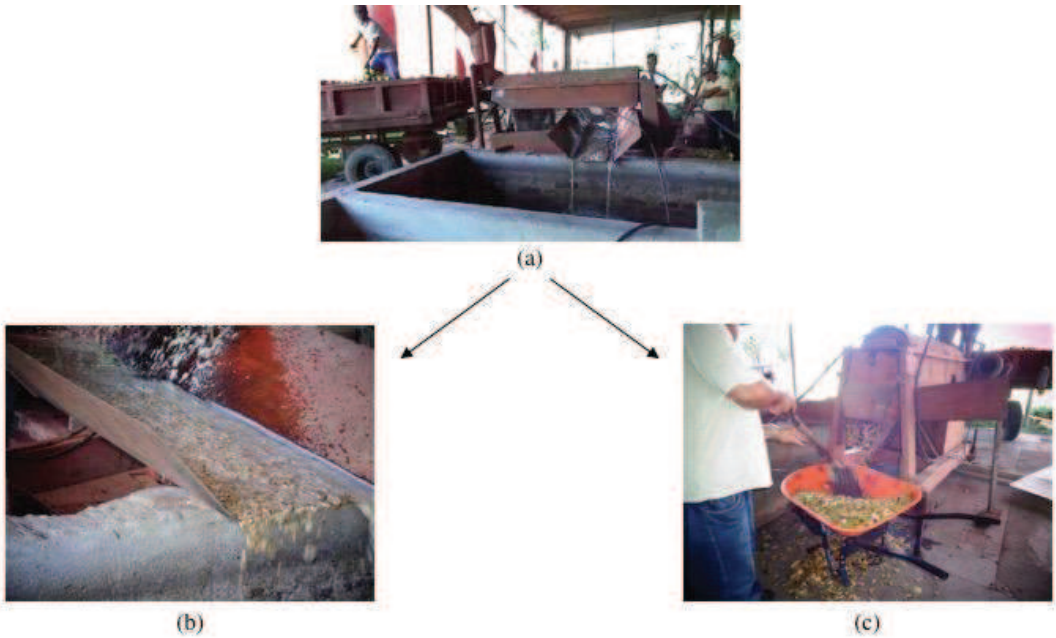

Figure 2.10. *Rupture of the fruits in the semi-traditional process. (a) Feeding the fruits in the crusher. (b) Extraction of the pulp and seed. (c) Separation of the shell*

Pulp and seeds fall into a pool of cement and more drinking water is added in order to allow the pulp to float, a step called flotation. This step has a duration of 8 to 12 hours and it requires an occasional stirring. The foam that is produced due to saponins, allows the physical separation of the pulp, as the pulp is attached to the air bubbles and

then carried to the surface where it is removed. Seeds settle out at the bottom. Alcoholic fermentation odor is easily perceived during this step. Nonetheless, the pulp is quite viscous initially and becomes more fluid during this step and this might be attributed to endogenous enzymes that help to liquefy the pulp.

The seeds are rinsed with drinking water several times and settled down. This process is repeated until the water remains colorless and clean (usually after 3 or 4 repetitions).

2.5.2.4 Flotation

This step is performed with a flotation-sieving technique. In the semi-traditional process, a molasses solution (58 °Brix) is used as the flotation medium. The seeds are immersed in a molasses solution (1.276 g/ml); the density difference allows the seeds to float on the surface and their separation is easy performed with a sieve. Seeds acquire a brown color due to the contact with the molasses solution. It is necessary to wash them with drinking water at constant flow for 20 min in order to remove it.

2.5.2.5 Drying

Dehydration is a useful mean to increase the shelf-life of seeds for further use. The open sun drying method is used by both processes. The seeds are placed on a concrete slab or sailcloth to dry under the sun for 4 hours at a temperature of about 38 ºC (see **Figure 2.11**). It is a simultaneous heat and mass transfer operation in which moisture is removed from the seeds and carried away by hot air. Seeds are dehydrated to a moisture content of 5 - 6%.

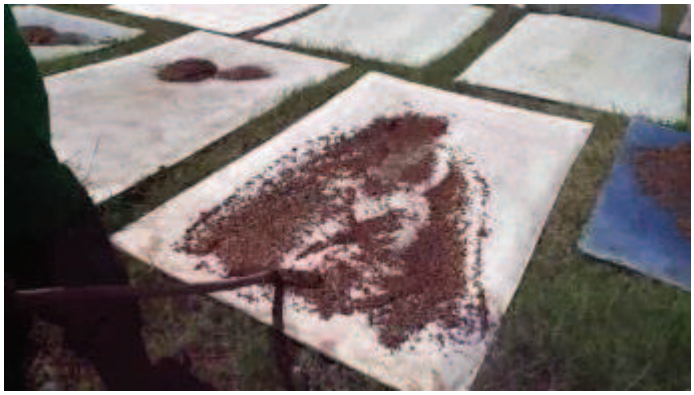

Figure 2.11. *Jicaro seeds drying under the sun*

2.5.2.6 Storage

The seeds are stored in polyethylene bags in a dry environment under suitable conditions of humidity, light and temperature for its conservation until its commercialization. They can be stored in these conditions for several years.

2.5.3 General view and comparison

The differences between the traditional and semi-traditional processes are shown in **Table 2.5**. The main difference between both processes is the storing time, which has a significant impact on the color of the seeds, lighter in the ones obtained with the semi-traditional one, probably because the fruit is not completely ripen.

Table 2.5. Comparison of traditional and semi-traditional processes of jicaro seed

Steps	Traditional process	Semi-traditional process
Harvest of fruits	Yellowish	Yellowish
Transportation	Wheelbarrow	Tractor trolley
Store time (days)	8	5
Breaking	Handmade	Machine
Extraction of pulp	Handmade	Machine
Soaking of pulp	12 hours	-
Squeezing of pulp	Machine/handmade	Machine
Soaking	-	8 – 12 hours
Washing	Water	Water
Flotation	-	Molasses solution
Drying	Under sun for 4 hours	Under sun for 4 hours

2.6 Oleaginous

2.6.1 Overview

Oleaginous are plants from which oil can be produced. The main source of oil is either the seed (oilseeds) or the fruit (mainly nuts).

A typical oilseed includes two basic parts: the seed coat and the kernel, which consist of two components, the embryo or germ and the cotyledons, which store the reserve nutrients (e.g. lipids, proteins and carbohydrates) and other compounds such as vitamins, phytosterols, phenolic compounds and minerals. Some examples of seeds rich in oil are sunflower seeds, sesame seeds, flax seeds and pumpkin seeds which are called oilseeds.

Edible nuts are fruits that usually have a hard, dry covering called shell that encloses an edible kernel (e.g. hazelnut, chestnut, walnut, almond, coconut). These can be consumed directly as food due to their characteristic flavors.

Other oleaginous which are neither oilseeds nor nuts include olive, avocado, corn, and palm among others.

2.6.2 Composition

The composition of oilseeds and nuts depends on the variety, the geographical origin, the cultural practices and the date of harvest (Sosulski and Sarwar, 1973). **Table 2.6** summarizes the nutritional value of some oilseeds.

Table 2.6. Nutrient content of some oilseeds (per 100 g of edible part)

	Cottonseed kernel	**Linseed/ flaxseed**	**Peanut (plain)**	**Rapeseed**	**Sesame seed**	**Soybean***	**Safflower seed**	**Sunflower seed**
Energy (kcal/kJ)	506/2117	492/2059	563/2337	452/1900	598/2470	141/590	517/2163	581/2410
Fat (g)	36.3	34.0	46	N	58.0	7.3	38.5	47.5
Saturated fatty acids	9.7	3.2	8.7	N	8.3	0.9	3.7	4.5
Mono unsaturated fatty acids	6.9	6.9	22.0	N	21.7	1.4	4.8	9.8
Polyunsaturated fatty acids	18.1	22.4	13.1	N	25.5	3.5	28.2	31.0
Carbohydrate (g)	21.9	34.3	12.5	8.3	0.9	5.1	34.3	18.6
Protein (g)	32.6	19.5	25.6	22.0	18.2	14.0	16.2	19.8
Fiber (as NSP unless specified) (g)	5.5	27.9	6.2	7.2 (crude fiber)	7.9	6.1	N	6.0
Vitamin E (mg)	N	0.3	10.1	N	2.53	1.13	N	37.77
Niacin equivalent (mg)	3.01	1.4	19.3	N	10.4	2.7	2.3	9.1
Folate (µg)	233	278	110		97	54	160	N
Sodium (mg)	25	34	2	5	20	1	3	3
Potassium (mg)	1350	681	670	800	570	510	687	710
Calcium (mg)	100	199	60	400	670	83	78	110
Phosphorus (mg)	800	498	430	800	720	250	644	640
Iron (mg)	5.4	6.2	2.5	N	10.4	3.0	4.9	6.4
Magnesium (mg)	440	362	210	250	370	63	353	390
Zinc (mg)	6	4.2	3.5	N	5.3	0.9	5.1	5.1

(*) boiled in unsalted water; N = traces were found. (McKevith, 1985)

2.6.2.1 Storage reserves

Seeds and nuts contain substances of nutritious reserves (stored food) for the development of the embryo. The major storage reserves are carbohydrates, lipids and proteins. Storage reserves are found in different parts of the seed called storage tissues such as the endosperm, the cotyledons (non-endospermic seeds) and the perisperm.

Among **carbohydrates**, starch is the main representative as the reserve substance of most seeds. It represents 65-70% of the dry weight in cereals. It is deposited as granules from 2 to 100 µm in diameter and located in cellular organelles called amyloplasts. Hemicelluloses, celluloses, glucans, pentosans and to a lesser extent free sugars such as glucose and sucrose, can also serve as energy source.

Lipids are the main source of energy in the germination of seeds. In oilseeds (18-45% of lipids) they are stored as triacylglycerol (TAG) in small spheres of 0.2 to 6 µm in diameter called lipid/oil bodies, oleosomes or spherosomes (Tzen, 2012). Lipid bodies are linked to some proteins called oleosins, where their main function is to bind the lipase enzymes, which are responsible for degrading the lipids during germination.

Seed oil bodies from diverse species are very similar in structure (Tzen et al., 1993). The matrix of triacylglycerol (TAG) is surrounded by a phospholipids layer embedded and covered with proteins, namely oleosin (15 to 26 kDa), caleosin (25– 35 kDa) and steroleosin (40 – 55 kDa), where oleosins are the dominant surface proteins (see **Figure 2.12**) (Jolivet et al., 2013)

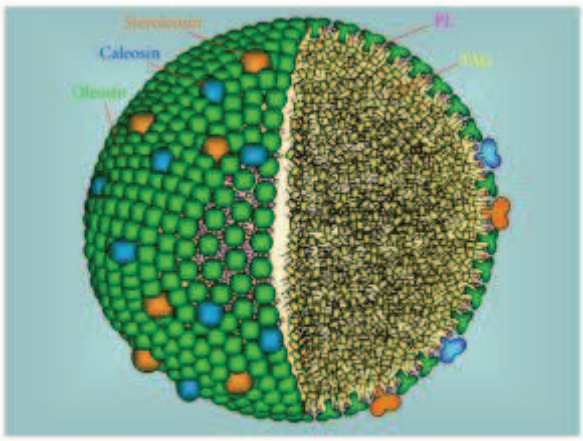

Figure 2.12. *A model of the oil body showing a triacylglycerol (TAG) matrix surrounded by a monolayer of phospholipids (PL) embedded with three classes of proteins, oleosin, caleosin, and steroleosin.*
(Tzen, 2012)

Oil content in oleaginous depends on the type of seed or nut as shows **Table 2.7**

Table 2.7. Average oil content in some oleaginous (oilseed and nut)

Oleaginous	Oil content (%)
Peanut	40 – 44
Coconut	60 - 68
Sesame	40 – 49
Niger	45 - 41
Safflower	35 -38
Sunflower	37 - 42
Linseed	33 – 42
Rapeseed	33 - 41.5
Soybean	19 - 21
Cotton	15 - 20

(Salunkhe et al., 1992)

Proteins represent about 8 to 15% of the dry weight of cereal grains and 40% in the seeds of legumes (Perissé, 2002). They constitute the main reserve of carbon and nitrogen for germination of the seedling.

Proteins are stored in organelles, which are often called protein bodies, with 0.1 to 0.25 µm of diameter. Several other terms such as protein vacuole, protein granule, and aleurone grain have also been used (Prakash and Rao, 1986).

Storage proteins found in protein bodies of diverse seeds have been classified into four groups, based on their solubility in various extraction solvents (Osborne, 1924):

- **Albumins**, which are water-soluble
- **Globulins**, soluble in diluted saline solutions
- **Prolamins**, which are soluble in alcohol
- **Glutelins**, which are diluted-acid or alkali soluble.

Albumins and globulins are widely distributed in dicotyledonous seeds, while prolamins are specifically found in cereals. Albumins act in the defense of the seed as inhibitors of trypsin and hemagglutinins. The globulins represent the most extensive distribution of storage proteins, being found in both monocotyledons and dicotyledons. The globulins have a similar structure and are classified according to their coefficient of sedimentation (e.g., 11S, 7S) (Shewry et al., 1995).

2.6.2.2 Other components

Apart from storage reserves, oleaginous also contain other significant components such as fatty acids, vitamins, minerals or polyphenols as well as anti-nutritional factors.

All oleaginous plants contain predominantly monounsaturated fatty acids (MUFAs) plus polyunsaturated fatty acids (PUFAs) (ranging from 74.65% for Brazil nut to 91.65% for pecan). Depending on the seed type, there is a significant variation in total MUFAs. Besides, seeds and nuts contain certain minerals in appreciable amounts as shown in the **Table 2.6**.

2.6.2.3 Anti-nutritional factors (ANFs)

It is known that seeds, nuts, and grains contain generally anti-nutritional factors (ANFs) (Liener, 1994; Mahajan and Dua, 1997; Venkatachalam and Sathe, 2006).

ANFs may be defined as substances generated in natural foods by the normal metabolism of species and by different mechanisms. Those compounds exert effects contrary to optimum nutrition since they reduce the availability of one or more nutrients and / or can cause toxicity (Kumar, 1992).

ANFs exist as a defense mechanism to protect the seed and the plant by discouraging insects, birds, and other predators from consuming them. Among these we can find enzyme inhibitors (trypsin and chymotrypsin inhibitors, plasmin inhibitors, elastase inhibitors), haemaglutinnins (e.g. concanavalin A, ricin), lypoxigenase, cyanogenic glycosides, oestrogens, saponins, gossypol from gossypium species e.g. cotton, tannins (e.g. condensed and hydrolysable tannins), amino acid analogues (e.g. mimosine, N-methyl-1-alanine), alkaloids (e.g. solanine and chaconine), anti-metals (e.g. phytates and oxalates), anti-vitamins (anti-vitamins A, D, E and B12) and favism factors (Aletor, 1993).

Some of these ANFs are temperature sensitive such as lectins and trypsin inhibitors; therefore they can be destroyed with thermic treatments (El-Adawy, 2002; Vidal-Valverde et al., 1994). Other compounds are more resistant to these temperatures (e.g. glucosinolates, phytic acid and tannic acid) (Mansour et al., 1993; Soetan and Oyewole, 2009).

Seeds and nuts are an excellent source despite their content of ANFs, as they can be removed with thermal treatments. This is why several techniques (e.g. boiling, roasting and extrusion) are usually applied for their destruction or reduction, allowing in this way to use them in human and animal feeding.

Soybean and soy-derived products are one of the most consumed oleaginous in the world, however, they contain many ANFs. Among them trypsin inhibitors, lectins,

phytic acids and indigestible oligosaccharides and phytoestrogens that may exert adverse effects on male fertility (Cederroth et al., 2010). The ANFs have limited their consumption, therefore they have to be appropriately treated prior to consumption. **Table 2.8** shows some ANFs present in several soybean genotypes.

Table 2.8. Anti-nutrients in soybean genotypes with high protein content

Genotype soybeans	Tannins (mg/g)	Phytate (mg/g)	Trypsin inhibitor activity (mg/g)	Saponins (mg/g)
SL 989	18.4	13.9	30.0	19.6
SL 992	12.1	18.0	50.1	20.8
PK 1026	16.6	18.7	56.5	19.8
Mean ± SD	14.4 ± 3.2	18.6 ± 2.6	53.3 ± 13.8	20.3 ± 0.6

(Sharma et al., 2013)

2.6.3 Uses of oleaginous

The main use of oleaginous is for the production of vegetable oils. The most important source of vegetable oils in terms of production are soybean, canola (oilseed rape), peanut, sunflower seed, cottonseed, palm kernel and copra (coconut oil). **Figure 2.13** shows the global oilseed production in 2015/1016. Global consumption of oils/fats is forecast to reach around 211 million tones in 2015/16 (FAO, 2016).

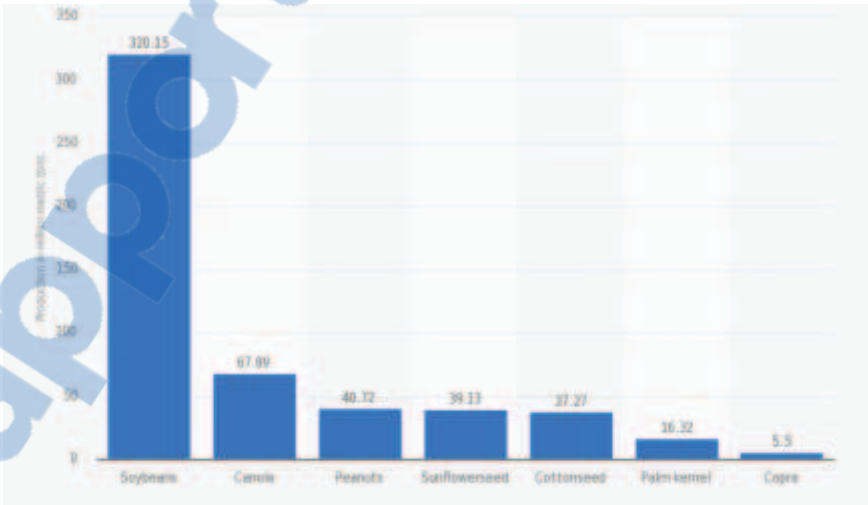

Figure 2.13. *Worldwide oilseed production in 2015/2016 (in million metric tons)*
(Statista, 2015)

The most consumed oleaginous are the following:

Soybean crop is the most widespread worldwide. The global consumption of soybean oil is 25% and of soybean meal is 65 % (Theones, 2006).

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Soybean is rich in nutrients mainly proteins and oil (40 - 20 % on dry matter basic) respectively. The quality of proteins is comparable to casein and whole egg (Sosulski and Sarwar, 1973). Isolated proteins from soybeans receive a score of 1 based on the amino acid reference pattern (Hughes et al., 2011).

The polyunsaturated fat content (73%) of soybeans is of interest as well because it includes linolenic acid (53.3 g/100), oleic acid (26.9 g/100) and α-linolenic acid (6.4 g/100) (Grela and Gunter, 1995).

Canola (oilseed rape) is now second only to soybean as the most important source of vegetable oil in the world. China and India dominate the production and consumption of this oil, and Canada is an important grower and exporter of seed. Like soybean, canola contains both high oil content as well as high protein content, with about 40% oil and 23% protein. Canola oil is often compared with olive oil, which is considered one of the healthiest oils. Both oils are rich in oleic acid (18:1), and both contain considerable amounts of polyunsaturated fatty acids such as linoleic acid (18:2) and linolenic acid (18:3) (Oplinger et al., 1989).

Peanut seed contains 25 to 32% protein (average of 25% digestible protein) and 42 to 52% oil. Its main use is for human consumption either as the whole seed or as processed products (peanut butter, oil, and other products) (Putnam et al., 1991).

Sunflowerseed varieties contain from 39 to 49% oil in the seed. Sunflower accounts for about 14% of the world production of seed oils. Sunflower oil is generally considered a premium oil because of its light color and high level of unsaturated fatty acids (oil oleic and linoleic) (Putnam et al., 1990).

Cottonseed is another crop used for oil production, although in a smaller scale. China is the major producer and consumer. This crop is mainly grown for its fiber; the seed oil being only a by-product.

Palm kernel is used to extract an edible vegetable oil. Malaysia and Indonesia are major producing countries. Palm kernel oil, palm oil, and coconut oil are three of the few highly saturated vegetable fats.

Copra oil is produced from the dried coconut flesh. It is made up of four medium chain triglycerides called MCT (C6 caproic acid, C8 caprylic acid, C10 capric acid, and C12 lauric acid). Coconut oil is about 50% lauric acid used equally by the food and oleochemical industries (Gunstone, 2011)

The quality of vegetal proteins has been extensively studied, specially soybeans, nuts and other seeds from non-conventional sources (Hughes et al., 2011; Jitngarmkusol et al., 2008; Moodley et al., 2007; Vadivel and Janardhanan, 2001; Wang et al., 2015).

The international market for products made from oleaginous is divided into three main segments: **oil, meal and raw material** (seeds or nuts). International oleaginous trade accounts for a consistently high share of the oilseeds global production, with around 31%. The countries of America, mainly the United States and Brazil, are the main producers of oilseeds followed by Asia (see Figure **2.14**) (OCDE-FAO, 2015).

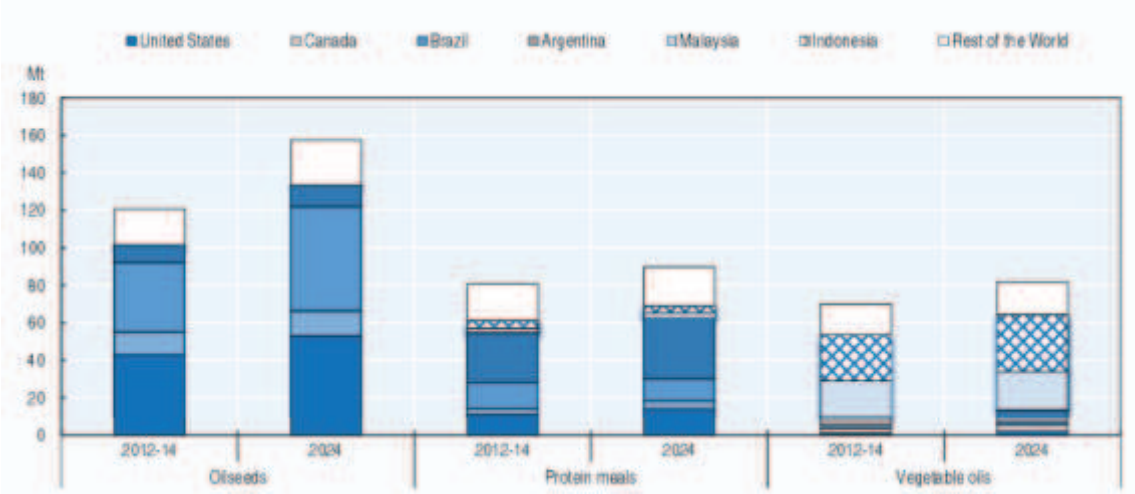

Figure 2.14. *Exports of oleaginous and oleaginous products by region (OCDE-FAO, 2015)*

Oil

Vegetable oils are mainly used as a human food. However, vegetable oils are also being used in biofuel production at different scales and using different methods of manufacture (Canakci and Gerpen, 2001; Fukuda et al., 2001; Stephenson et al., 2008).

Oil extraction involves operations of crushing of the oilseeds and usually heating to inactivate enzymes. There are two systems used for oil extraction from oilseeds, one purely mechanical and another one which also uses solvents. In both systems, the seeds must be previously cleaned, husked, and ground (Schukla et al., 1992). After the oilseed extraction process, the residue can be used as a source of protein (35 % -60 % d.b.).

Meal

The oleaginous meal is a by-product of oil production, namely the residue remaining after oil extraction. Oleaginous meals from soybean canola, sunflower, cottonseed are good sources of protein, fiber, vitamins and minerals widely available in

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LE NUMERO 1 MONDIAL DU MÉMOIRES

the market (Sarwar, 2013). Protein content of defatted meals is between 35% and 60% (d.b.). Some oilseed meals have proteins comparable to casein and whole egg in distribution of amino acids (Sosulski and Sarwar, 1973).

Some of oleaginous meals are used to manufacture valuable products for use in ruminant, swine, poultry, aquaculture and animal diets (Ravindran and Blair, 1992; Rutkowski, 1971; Williams, 1995).

Seeds and nuts

Many oleaginous can be found in the market as unprocessed products. 20% of men and women consumed nuts and seeds during the 7-day survey period of the recent National Diet and Nutrition Survey (NDNS).

They are usually transformed later into oils or meals, but in some cases (e.g., peanuts) can be consumed in this form or with minimal transformation (roasting).

Vegetable milks

In some specific cases, namely soybean, peanut, coconuts and other nuts such as almonds, hazelnuts or walnuts, vegetable milks are manufactured from the whole oilseed.

The most outstanding characteristic of the vegetable milks are lactose-free and cholesterol-free. In addition, these are a good source of minerals mainly calcium and phosphorus and B-complex vitamins. Soy milk is the most consumed vegetable milk, which provides essential amino acids, lecithin, omega-3 fatty acids and estrogens such as isoflavones.

Among the different techniques used in the processing of oleaginous plants, thermal treatments in general, and specifically roasting is one of the most important ones. The advantages are multiple, including the (sometimes partial) removal of ANFs, the increase in the performance of the oil extraction or the improvement of their organoleptic properties.

2.7 Roasting technology

2.7.1 Overview

The basic purpose for the thermal processing of foods is to reduce or destroy microbial activity, reduce or destroy enzyme activity and to produce physical or

chemical changes to make the food meet certain quality attributes (flavor, texture, color and as well as reductions in heat-sensitive nutrients) (Heldman and Hartel, 1998).

Heat treatments include mild processes such as blanching and pasteurization or more severe processes as cooking, frying, baking, roasting and sterilization (canning) (Safefood 360, 2014) .

Roasting can be defined as a dry heat treatment or processing technique that is used to either cook or pre-cook food materials with an open flame, oven or other heat sources. It involves the simultaneous transfer of heat into the food and the removal of moisture by evaporation from the food to the surrounding air (Fellows, 2000).

Roasting and baking are considered the same unit operation because they both use heated air to alter the quality of foods (Fellows, 2000). According to Fellows (2000), baking is usually applied to flour-based foods or fruits, and roasting to meats, nuts, grains and vegetables.

Roasting is a time and temperature-dependent process (Amaral et al., 2006; Rosli et al., 1998; Yen, 1990). In most cases this process improves the flavor, color, and texture of coffee, walnuts, pumpkin seeds, cocoa and other products (Baggenstoss et al., 2008; de Brito et al., 2001; Kita and Figiel, 2011; Murkovic et al., 2004). Roasting is also performed to remove the seed coats of the kernels, inactivate enzymes, destroy microorganisms and reduce water activity.

Most of the changes in product quality attributes can be described mathematically using first-order kinetics.

2.7.2 Methods

The three main methods of heat transference used in roasting are the following (Marcotte, 2007):

- **Convection**: It is a heat transfer mechanism that results from the movement of particles originated either by density gradients set up by temperature differences or forced by external elements such as fans. Newton's law of cooling models this mechanism. It states that *the rate of convective heat transfer is proportional to the surface area of heat transfer and the temperature difference between the surface and the fluid*:

$$Q = hA\,(T_s - T_f)$$

where Q is the heat transfer by convection, h is the heat transfer coefficient, A the product surface, T_s is the product surface temperature and T_f is the temperature of the bulk fluid.

- **Conduction**: It is a heat transfer mechanism that occurs within bodies and is transmitted directly through atoms or molecules, which interact with neighbor particles transferring their kinetic and potential energies. It is modeled by the Fourier's law of heat conduction, which states that *the heat transfer rate through a uniform material is directly proportional to the area of heat transfer and the temperature gradient with reference to the thickness in the direction of heat transfer*:

$$\frac{dQ}{dt} = -kA\frac{dT}{dx}$$

  where dQ/dt is the rate of heat transfer, A is area of heat transfer, dT/dx is the temperature gradient across unit thickness and k is the proportionality constant (thermal conductivity). A negative sign is included on the right-hand side because dT/dx is negative (temperature decreases as thickness increases).
- **Radiation**: It is an energy transfer mechanism in the form of electromagnetic waves. All bodies emit electromagnetic waves at wavelength between 0 and infinity. Radiant energy travels uninterrupted until it strikes a surface where it is absorbed, reflected or transmitted. Radiant energy is transmitted through electromagnetic waves and is released after impacting a contact surface, converting into heat.

2.7.3 Types of roaster

Based on the previously mentioned heat transfer mechanisms, many roasters have been developed. In this section, we describe the ones that are mostly employed for the roasting of seeds.

2.7.3.1 Batch roaster

It is a convection-based system that consists of: a) a hopper for feeding the material; b) a roasting chamber or drum through which hot air passes and the material gets mixed; c) a discharge unit that unloads the roasted material at the end of roasting and d) a driving motor.

A batch roaster with recirculation of air is also possible by employing a furnace that supplies the heated air. A blower delivers air to the burner in the furnace and suitable valves control the rate at which fuel and air flow is needed. A cyclone is installed for collecting the chaffs and husks that are generated during roasting.

2.7.3.2 Continuous roaster

Continuous systems (inclined horizontal systems and spiral tube systems) are employed for large-scale manufacturing of roasted grains. The feeding of the raw material is a constant flow rate. Materials are heated in the spiral tubes directly for a desired residential time and temperature, then the material is gradually roasted and transported to the cooling chamber by vibration. Therefore they are based on a wide range of products can thus be roasted or toasted, such as almonds, cashew nuts, pistachios, hazelnuts, sunflower seeds, pumpkin seeds, sesame seeds, wheat, barley, tea and spices. (Bhattacharya, 2015).

2.7.3.3 Fluidized-bed roaster

Developed in the 1970's by Michael Sivetz. In fluidized beds, the hot air flows through a chamber providing a homogeneous distribution of heat. The hot air is blown through the bed, causing the food to become suspended and vigorously agitated (fluidized), exposing the maximum surface area of food.

The time for roasting mainly depends on the depth of material, moisture content and temperature of roasting. However, this technology is considered as ultra-fast roasting due to roasting times range from 30 s to 3 min (Price et al., 1991).

2.7.3.4 Microwave roasting

It is the quickest method of heat treatment of food materials, because the time of roasting is ten times faster than a conventional cooking method saving time and electrical energy as observed in almonds (Agila and Barringer, 2012). The effects of microwave heating on different vegetable have been investigated. This studies indicate that nutrient retention during microwave processing is not much greater than that in conventional cooking (Yoshida et al., 2002; Yoshida and Kajimoto, 1994).

2.7.4 Physico-chemical changes induced by roasting

2.7.4.1 Physical changes

The main changes of roasted grains are evident in terms of weight, density, moisture, color and flavor (Agila and Barringer, 2012; Hernández et al., 2007; Jinap et al., 1998; Mwithiga and Jindal, 2003).

Among microstructural changes there have been observed crispness and crunchiness (Young et al., 2004) and distortion of the subcellular organization (Dean et al., 2014; Saklar et al., 2003). These changes are related to changes in the texture. These are determined by the nature of the food (moisture content and the composition of fats, proteins and structural carbohydrates) and by the temperature and duration of roasting. When the surface dries, the texture becomes crisper and harder as a porous crust is formed by coagulation, degradation and partial pyrolysis of proteins. In cereal foods, changes to the granular structure of starch, gelatinization and dehydration produce the characteristic texture of the crust (Fellows, 2000).

Popping and puffing both involve a sudden expansion of water vapor when grains are subjected to high temperature for a short time during roasting. During popping of cereals (e.g. corn, sorghum, rice) the starch granules contained in the endosperm are gelatinized and expand by heat, giving rise to a three-dimensional network.

The popping quality, including volume expansion, is influenced by the moisture content of the grain and the time and temperature of roasting (Konishi et al., 2004; Sharma et al., 2014). In the case of corn a moisture content between 10 - 15% gives the maximum expansion at 177 ºC (Hoseney et al., 1983).

Another example is the paddy rice where the maximum popping was observed at 14.94 % of moisture content at 850 W and heating period of 40 s. In addition, the grain hardness, husk content, husk interlocking, hydration capacity are quality parameters for popped rice. **Figure 2.15** shows quality of microwave popping of paddy.

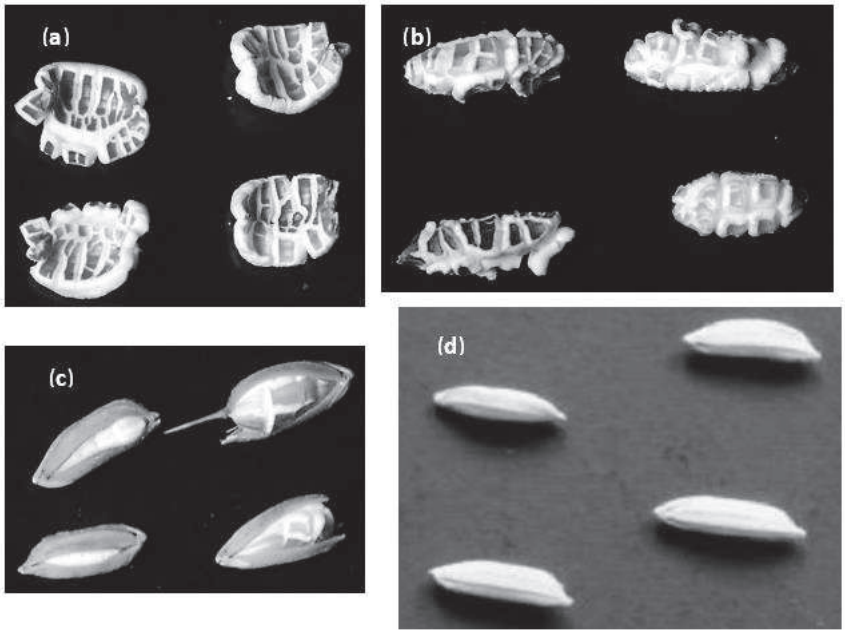

Figure 2.15. *Different states of popping of paddy using microwave energy. a) Fully popped with open structure; b) Popped without rupture in shape; c) popped with improper expansion and d) Un-popped paddy*
(Kumar Swarnakar et al., 2014)

Popping or puffing of grains is conducted by oven, gun puffin, roasting with sand, oil, salt or hot air. The mode of that transfer is by conduction when using sand, salt or even a hot metal surface; by convection using hot air, radiation (i.e., infrared, microwave) or a combination of the type of heat transfer. High-temperature puffing shows bigger and more uniform air cells. Low temperature gives non-uniform and small air cell (Konishi et al., 2004).

2.7.4.2 Chemical transformations

The more extensive and complex chemical reactions during roasting include the Maillard reaction, the Strecker degradation, pyrolysis and caramelization, mainly resulting in aroma, flavor and color compounds.

The Maillard reaction is divided into three stages, as depicted in **Figure 2.16** (Mottram, 2007). The initial stage involves the condensation of the carbonyl group of the reducing sugar (aldose) with the amino compound to form glycosylamines and/or aminoaldoses and/or aminoketones by condensation.

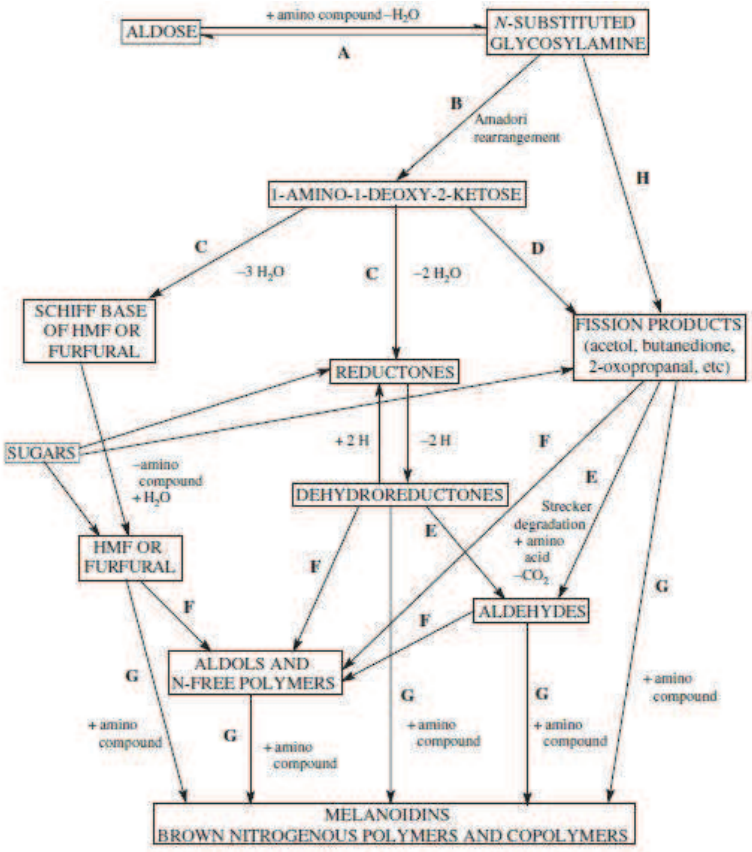

Figure 2.16. *Schema of the Maillard reaction.*
(Badui, 2006)

In the intermediate stage of the Maillard reaction, sugar dehydration produces reductones and then the Strecker degradation takes place, where Strecker aldehydes and ketones are generated, along with a set of rearrangements and dehydrations which produce volatile compounds. In this stage, there is an initial formation of light yellow colors.

In the final stage, brown nitrogenous polymers are produced, called melanoidins. It also involves the formation of flavor compounds, mainly heterocycles.

The Maillard reaction is of great importance for flavor formation. The volatile compounds produced in the reaction are sugar dehydration or fragmentation products (e.g., furans, pyranones, furanic compounds, carbonyl compounds, acids); amino acid degradation products (e.g., aldehydes, sulphur compounds, nitrogen compounds); and volatiles produced by further interactions (e.g., pyrroles, pyridines, pyrazines, alkylpyrazines imidazoles, oxazoles, thiazoles, thiophenes) (Mottram, 2007).

Pyrazines are the most important volatile compounds in roasted products. They contribute to the pleasant and desirable flavor in cocoa beans, coffee, nuts, etc. The

number of pyrazines increases with the roasting time and temperature (Jinap et al., 1998).

The Maillard reaction is one of the most well-known nonenzymatic browning reactions in heat-treated food products. The reaction produces a diverse collection of products ranging in molecular weight from simple gases like CO_2 to complex high molecular weight polymeric compounds known as melanoidins which provide the brown color to the cooked food. But color is a function of time and temperature of heat treatment (Özdemir and Devres, 2000). Melanoidins are formed by cyclizations, dehydrations, retro aldolizations, rearrangements, isomerizations, and condensations of low molecular weight of the Maillard reaction products (MRPs) (Mottram, 2007).

Colored Maillard reaction products (CMRP) have been isolated from model Maillard reaction systems (Rizzi, 1997). Chromophore structures of low molecular weight (<500 MW) with a high conjugation level were isolated from model systems using different sugars and amino acids (Hofmann, 1998).

On the other hand, roasting leads to protein denaturation and it could also improve lipid extractability in oilseeds (Amaral et al., 2006; Chirinos et al., 2016; Yoshida and Kajimoto, 1994).

2.8 Dehulling (decortication)

The seed coat (hull) is the surrounding layer around the cotyledons and the endosperm. It has a very important function in the nutrition and development of the embryo, since it serves as protection against harmful agents in the environment.

In the most cases the seed coat must be removed to improve its nutritional value and organoleptic characteristics and in some cases (e.g. oilseeds) improve oil recovery (Inyang and Iduh, 1996).

The chemical composition of the seed coat is generally very low in protein and high in fiber, mostly indigestible fiber (e.g. cellulose, hemicellulose and lignin). Hulls provide dark colors to the meal (Brillouet and Riochet, 1983; Johnson et al., 1979). Its proportion varies from oilseed to oilseed as shown in **Table 2.9**. The hull contains large amounts of oxalic acid and polyphenolic compounds (e.g. tannins, proanthocyanidins) (Amarowicz et al., 2000; Brillouet and Riochet, 1983; Choung et al., 2001; Johnson et al., 1979; Li et al., 2008).

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Table 2.9. Approximate proportions of hulls and kernel in some oilseeds

Oilseeds	Kernel (%)	Hull (%)
Groundnut	75	25
Rapeseed mustard	80-82	18-20
Soybean	93	7
Sunflower	60-70	30-40
Safflower	50	50
Castor	70-80	20-30
Cottonseed	62	38
Sesamum	82-86	14-18
Linseed	57	43

(Schukla et al., 1992)

Dehulling or decortication is the loosening and removal of the seed coat to produce a polished seed (Wood and Malcolmson, 2011). The removal of the seed coat is beneficial for the following reasons:

- It improves the nutritional quality, such as protein digestibility, as it reduces the amount of antinutritional factors, such as tannings and insoluble fiber.
- It improves texture and palatability, as it removes the astringent taste caused by tannins.
- It improves the general appearance, as the resulting products are lighter in color (e.g, white flours).

Decortication operations consist of two steps: the cracking of the seeds into cortices (hull) and kernels (cotyledons) and thereafter their separation (Hoffmann, 1989). Some hulls are hard or fibrous, so it is necessary to roast or boil the seeds to remove the hull without breaking the seed. Dehulling can be done before or after extraction of the oil from the seeds either by a front or tail-end dehulling.

2.8.1 Mechanical dehulling

Mechanical dehulling can be done by friction or abrasion procedures. Mechanical dehulling is the procedure used in flaxseeds, sesame seeds and sunflower seeds (Kaya and Kahyaoglu, 2006; Subramanian et al., 1990).

Different procedures and machines have been employed for mechanical dehulling (Hoffmann, 1989):

- **Knife dehuller**: This machine consists of a horizontal rotating cylinder fitted with protruding longitudinally placed knives or bars. Surrounding

the rotating cylinder for part of its circumference there is a concave section fitted with opposing knives. The seeds are cut between the cutting edges as the cylinder rotates.

- **Disc dehuller**: This machine houses two vertically mounted disc with radial grooves, one rotating, one stationary. The seeds are fed to the center of the disc, are broken by the grinding action, and then discharged centrifugally to the periphery of the disc.
- **Impact dehuller**: These machines generally consist of a rotor made up of paddles, bars or tubes positioned at the circumference of the reel, which fling the seeds against a corrugated impact plate covering about half of the circumference. The machines are uniformly fed at the top by different types of feeders (e.g. fluted roll), whereas the mixture of cut and uncut seeds plus husks leaves at the bottom.
- **Tangential abrasive dehulling device (TADD)**: It consists of a single abrasive stone on the horizontal plane. This machine has been used for many types of grains such as sorghum, wheat, corn and amaranth grain. It has also been applied in oilseeds as flaxseed (Oomah and Mazza, 1993).
- **Rubber roller decorticator**: It consists of two rubber rollers rotating in opposite directions and speeds with a blower at the base of the chamber. The roller presses the seed coat and releases the seed kernel; the mixture of both seeds and seed coats passes through a sieve into the chamber, where the lighter seed coat gets blown away, keeping the seed kernel in the chamber (Puri, 2005).

2.8.2 Chemical dehulling

Chemical dehulling is usually employed in combination with mechanical dehulling as a mean of favoring the removal of the seed coat. Both alkaline substances (e.g. KOH, NaOH, $NaHSO_3$) and acid minerals (e.g., H_2SO_4, HCl) are used. The main drawbacks of these techniques include the decrease in the nutritional value of the grains, the impact on their flavor and color and the need of large volumes of water in the final phase in order to remove the chemical substances used in the procedure.

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LE NUMERO 1 MONDIAL DU MÉMOIRES

For instance, chemical dehulling has been used for corn grains, however it has been proved that it decreases their nutritional content and affects their color (Blessin et al., 1970; Carbonell-Barrachina et al., 2009; William, 1950).

Chapter 3

Materials and Methods

3.1 Raw material

3.1.1 Origin

Jicaro seeds (*Crescentia alata*) were obtained during two harvesting seasons in November 2013 and November 2015. A total of 200 kg of dried seeds were taken, gathered and mixed at the Universidad Nacional Autonoma de Nicaragua (UNAN-León). The seeds were washed again, sun dried and the foreign material (e.g., gravel, straw, hard shell fragments) was removed. All analyses were performed on samples of these representative batches.

3.1.2 Samples

The characterization was performed on different parts of the seed. All samples were ground in PREP'LINE knife blender (SEB, Ecully, France) and they were analyzed before and after roasting.

The following samples were used:

Whole seeds: formed by the embryo, cotyledons and seeds coat.

Cotyledon (kernels): is part of the embryo within the seed, which they act as reserve carbohydrates, lipids and proteins.

Seed coat: outer protective coating of seeds.

Additionally, soybeans were used as a comparison reference

Soybeans: commercial hulled yellow soybean (origin: Markal, Saint-Marcel-lès-Valence, France).

3.2 Physico-chemical analysis

3.2.1 Moisture

Moisture content was determined by using a drying oven at 105°C for 6 hours according to NF ISO 6496 standard (ISO, 1999). The samples were weighed (2.5 g) into a capsule, then were placed into the drying oven for 6 hours until reaching a stable weight. After this time, the capsule was removed from the oven and placed directly into a desiccator and cooled. The capsule with the sample was weighed. The moisture content was calculated with the following equation:

$$\text{Moisture (\%)} = \frac{m_i - m_d}{m_s} \times 100$$

Where: m_i is the initial mass (g) of the wet sample; m_d is the final mass (g) after dehydration and m_s is the mass of the wet sample (g).

3.2.2 Crude protein

Protein content was measured by Kjeldahl's method AOAC (1999), which evaluates the total nitrogen content of the sample after being digested in sulphuric acid, which decomposes the organic substance by oxidation to liberate the reduced nitrogen. The sample was weighed (0.3g) and placed in a digestion flask and then digested by heating it (420 °C for 45 min) in the presence of 12 mL concentrated sulfuric acid, 5g potassium sulphate K_2SO_4, 0.15g of copper (II) sulphate $CuSO_4.5H_2O$ and 0.15 titanium dioxide TiO_2 (one catalyst tablet). After the digestion, has been completed the flask was connected to a distillation unit (Tecator ™ Line 8400, Höganäs, Sweden, Dk). The solution in the digestion flask was then made alkaline by the addition of sodium hydroxide, which converts the ammonium sulfate into ammonia gas. The nitrogen content is then estimated by titration of the ammonium borate. The determination of protein content was calculated with the following equation:

$$\text{Protein (\%)} = \frac{g_{N_2} \times N_{H_2SO_4} \times ml_{H_2SO_4} \times 6.25}{1000 \times g_{\text{sample}}} \times 100$$

Where g_{N_2} are grams of nitrogen, $N_{H_2SO_4}$ is the normality of the acid, $ml_{H_2SO_4}$ is the volume consumed during titration, the value of 6.25 is the conversion factor and g_{sample} is the mass of the sample in grams.

3.2.3 Crude fat

It was carried out by a direct method for continuous extraction with a solvent. Two techniques were used.

By a Soxtec system. The content of fat was measured using an extraction unit Soxtec ™ (Höganäs, Sweden). Samples (0.5g) were ground and placed directly in the thimble and immersed in 50 ml of boiling solvent (diethyl ether) into cups previously tared (m_1). Then, they were raised above the solvent surface to permit an efficient washing of the sample with solvent from the condensers. The extraction cups were taken and placed in the oven for 1h at 60 ºC. Then they were removed from the oven and placed into desiccator and then they were cooled for 45 min. The cups were reweighted (m_2). The determination of crude fat was calculated with the following equation

$$\text{Crude fat (\%)} = \frac{m_2 - m_1}{W} \times 100$$

Where m_1 is the mass of the empty cup and; m_2 is the mass of the cup with the fat extraction and W is the mass of the sample

By an accelerated solvent extraction system (ASE DIONEX Corp., Sunnyvale, USA). Samples were ground and weighed (1g) and then introduced into a ASE™ 350 cell. The ASE™ 350 system consists of a stainless-steel sample cell with electronically controlled heaters and pumps to maintain extraction parameters (pressure, temperature, volume of extraction solvent, extraction time) at the programmed set points. Flush was set to 100%, number of cycles to 5 with a static time of 7 min, and purge duration was 90 sec. The extraction solvent containing lipids was collected in a 50 mL flask that had been previously dried and weighed. Quantification of fat was made gravimetrically after solvent evaporation. Lipid content was expressed as g fat per 100 g sample (dry weight basis).

3.2.4 Ash

Ash content was measured by using a muffle furnace (Thermo Scientific™) according to AOAC method 923.03 (AOAC, 2000). About 0.5 g of sample was placed

in a porcelain crucible and subsequently placed in a muffle furnace at 550°C for 2 hours. After 2 hours, the crucible was removed from the furnace and placed directly into a desiccator and cooled for 30 min. The crucible was reweighted. Ash content has been performed in triplicate experiments. Ash content was calculated with the following equation:

$$Ash\ content\ (\%) = \frac{W_3 - W_1}{W_2 - W_1} \times 100$$

Where W_1 is the weight of crucible; W_2 is the weight of crucible and sample and W_3 is the weight of crucible and ash.

3.2.5 Fiber

Samples were analyzed in the Mediterranean and Tropical Livestock Systems, SELMET- UMR, France. The fiber content was determined by Van Soest technique (Van Soest, 1963).

The sample was successively subjected to the action of neutral detergents and acids and finally to the action of concentrated sulfuric acid. 1g of sample was weighed directly into the filter crucible. The sample was ground and defatted with petroleum ether. The crucible containing the sample was placed in the fiber extractor (Fibertec™ Systems).

For neutral detergent fibers (NDF), a neutral detergent solution (sodium lauryl sulfate, USP-grade and ethylenediaminetetraacetic acid, EDTA; pH 7) at boiling temperatures with a 0.2 mL of heat-stable α-amylase Termamyl® (Laboratoires HUMEAU, La Chapelle-sur-Erdre, France) was used to dissolve the easily digested pectins and cell contents (proteins, starch sugars, and lipids), leaving a fibrous residue NDF (cellulose, hemicellulose and lignin). For acid detergent fibers (ADF), 100 mL of an acid detergent solution (20 g cetyl trimethylammonium bromide and 0.5 M H_2SO_4) were used to dissolve hemicellulose and minerals. Finally, for acid detergent lignins (ADL), a 3h digestion was performed with 72% H_2SO_4 in a crucible. Waste mineralization was performed at 550 °C for 4 h. The results are reported on a dry matter basis, as cellulose (ADF-ADL), hemicellulose (NDF-ADF) and lignin (ADL). The calculus was performed with the following equations

$$\text{NDF}\ (\%) = \frac{m_{\text{ndf}} - m_1}{W} \times 100$$

$$\text{ADF (\%)} = \frac{m_{\text{adf}} - m_1}{W} \times 100$$

$$\text{ADL (\%)} = \frac{m_{\text{adl}} - m_1}{W} \times 100$$

Where W is the weight of sample (g) and m_{ndf} is the mass in grams of the crucible containing the residue NDF + ashes and m_1 is the mass of the crucible.

3.2.6 Sugars

A sample (1 g) and 0.5 g of sand were introduced into a stainless-steel extraction cell (5 mL capacity, Dionex™ ASE™) which was positioned into the carousel ASE 350. The numbered vials (ordered according to the cell) were placed on the lower carousel of the ASE 350. For the extraction of soluble sugars, the programming profile was as follows: a 80% ethanol solution (v/v) was used as the solvent at 60°C, 100 bar with 3 cycles. After extracting, the cells were cooled at least 15 minutes before handling. Sugar analysis was performed on the lipid-free meal remaining in the extraction cell.

Extracts of recovered sugars were diluted 25-fold with deionized water and filtered to 0.45 µm before injection of 10 µL into the chromatograph. Soluble sugars (i.e. fructose, glucose and sucrose), were separated using high performance ionic chromatography (HPIC) with a DX600 system equipped with a Carbopac MA-1 column (250 x 4 mm), a Carbopac MA-1 guard column (25 x 4 mm) and a Dionex ED50 pulsed amperometric detector (PAD) (DIONEX Corp., Sunnyvale, CA, USA). Chromatographic conditions were described by Valente et al. (2013).

The determination of α-galactosides (raffinose, stachyose and verbascose) was performed according to a procedure described by Muzquiz et al. (1999). A sample containing phenyl α-D-glucoside (100 µg) as an internal standard and 48% aqueous ethanol was added. Extraction was performed using a sonication for 60 min, followed by centrifugation at 700×*g* for 10 min. Combined supernatants were heated at 85°C under reflux for 30 min, cooled and centrifuged at 700×*g* for 5 min. The supernatant was evaporated to dryness. The residue was dissolved in water, and an aliquot was transferred into a glass-stoppered test tube. Acetonitrile (1.0 mL) was then added with shaking, and the mixture was stored overnight at 4°C. The sample was injected into an HPLC DX600 system (DIONEX Corp., Sunnyvale, CA, USA).

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LE NUMERO 1 MONDIAL DU MÉMOIRES

3.2.7 Mineral composition

Mineralization (500 °C) of the sample in an ash furnace (Thermo Scientific™ Thermolyne™ 6000 series 408, Waltham, Massachusetts, USA) was performed prior to the analysis of P, K, Na, Ca, and Mg and similarly for trace elements (Fe, Mn, Cu and Zn) until the ashes were cleared. Ashes were then digested with hot concentrate hydrochloric acid until the destruction of organic matter, as described by Pinta, (1973) methodology. Mineral contents were performed by inductively coupled plasma atomic emission spectroscopy ICP - AES) (Varian Vista-Pro, Palo Alto, CA, USA) and quantified against standard solutions of know concentrations.

3.2.8 Phytate determination

Phytate content was measure as describe by Sekiguchi et al., (2000). Jicaro seeds (0.1 g) were placed in 2 mL 0.5 M HCl overnight at room temperature with constant stirring. After centrifugation (10 min, 10,000×*g,* 10 °C), the supernatant was recovered and then diluted in 0.2 M borax buffer (pH 8.0). Phytate content (inositol hexakisphosphate, IP6) was measured using HPIC with a DX600 system, equipped with an ATC-1 trap column, an AG11 guard column and an AS11 column. Detection was performed with a conductivity cell connected to an ED50 detector after removing anions on an ASRS 300 (DIONEX Corp., Sunnyvale, CA, USA). The injected volume was 10 µL. Phytate elution was performed using a basic gradient of 200 mM NaOH. The gradient was linear from 30 to 80 mM NaOH for 8 min, before returning to and holding initial conditions for 8 min to re-stabilize the system. Data were analyzed with Chromeleon 6.0 software (DIONEX Corp., Sunnyvale, CA, USA). Calibration was done by using an external IP6 standard.

3.2.9 Phenolic determination

Total phenolic content was evaluated at 760 nm with the Folin-Ciocalteu reagent as described by Singleton, Orthofer & Lamuela-Raventos (1999). The lipid fraction was removed with hexane, and polyphenol extraction was performed on a 0.5 g sample added with 10 mL of acetone/water/formic acid (70/29/1, v/v/v). The results, in triplicate, are expressed as mg of gallic acid equivalents/100 g on a dry weight basis (mg GAE/100 g).

3.2.10 Tannins determination

Tannins were determined according to the ISO AFNOR NF V03-75 standard. They were extracted from a 1 g sample with dimethylformamide. After centrifugation (10 min, 10,000×*g*) and addition of iron (III) ammonium citrate, the absorbance of the supernatant was measured using spectrophotometry at 525 nm. The results are expressed as mg of tannic acid equivalents/100 g on a dry weight basis.

3.2.11 Tocopherols analysis

Tocopherols were extracted by using a method described by Taylor et al., (1976) and modified by (Deiana et al., 2002). A 0.5 g oil sample was added with 2 mL ethanol/pyrogallol (1%) and heated in a water bath at 70 °C for 2 min. Saponification was performed with 1 mL 12 N KOH for 30 min at 70 °C. Samples were extracted twice with n-hexane. Hexane phase was evaporated to dryness, suspended in absolute ethanol and filtered through a 0.45 µm micropore nylon membrane before injection. Tocopherols were analyzed according to the modified method described by Rossetti et al. (2010) using HPLC with a Dionex Ultimate 3000 system (DIONEX Corp., Sunnyvale, CA, USA) equipped with an Uptispher C_{18}-HDO column 250 mm x 4.6 mm, 5 µm (Interchim, Montluçon, France). The injection volume was 20 µl. The elution was performed isocratically with ethanol/methanol 60/40 (v/v) at a flow rate of 0.8 mL/min at 25 °C. Detection was performed with a fluorescence detector at 296 nm and 330 nm.

3.2.12 Fatty acid composition

Fatty acid composition was determined as described by Piombo et al. (2006). Oil samples (10 mg) were added with 3 mL sodium methylate solution with phenolphthalein in rounded bottom flasks. Hydrochloric methanol (3 mL) was added until phenolphthalein discoloration was observed. Hexane (8 mL) and water (10 mL) were added. The organic phase was recovered, dried over anhydrous sodium sulfate, and filtered for subsequent GC analysis using an Agilent 6890 chromatograph (Agilent, Santa Clara, CA, USA) equipped with an Innowax capillary column 30 m x 0.32 mm x 0.25 µm (Agilent, Santa Clara, CA, USA). Fatty acid methyl esters (FAME) were directly injected into the GC (He 1 mL/min, split 1:80). Injector and FID detector temperatures were 250 °C and 275°C respectively. The oven was heated from 185 to 225 °C at 5 °C / min and held at 225 °C for 20 min. Fatty acids were identified by

comparing their retention times with the FAME standards (Sigma-Aldrich, St. Louis, Missouri, USA).

3.2.13 Free amino acids analysis

Samples (15 mg) were ground and introduced into a vacuum hydrolysis tube of 1 ml with 50µl of norleucine as an internal standard. Complete removal of oxygen was ensured by connecting the hydrolysis tube to a vacuum pump and a nitrogen gas source, using a three-way stopcock to alternate between vacuum and flushing with gas (N_2). When all hydrolysates were released the tube were placed on a Reacti-Therm Heating/Stirring module (Pierce, Rockford, IL, USA) at 150°C for 120 min. After this time, the tubes were cooled for 5 min at room temperature and 450 µl of NaOH 4 N were added. The reaction medium was removed with a Pasteur pipette and placed in a 5 ml volumetric flask. The tube was rinsed 3 times with the dilution buffer Sodium citrate pH 2.2. The volume of the flask was adjusted to 5 mL with this buffer. Free amino acids were analyzed following the method used by Moore et al., (1958) Total amino acid analysis was performed using a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK). This system uses ion exchange chromatography with post-column ninhydrin derivatization and photometric detection with dual-wavelength measurements. The amino acid separation along the cationic column was obtained with a succession of four sodium citrate buffers of increasing pH (2.6–8.6) and ionic strength (0.2–0.5 M) and with an increasing temperature gradient (52–95 °C). Amino acids were derivatized with the ninhydrin reagent (135 °C) and detected simultaneously at 570 nm and 440 nm. The entire process lasted 90 min per sample, including the resin regeneration phase. Quantification was performed by comparing peaks areas with a complete standard including 26 amino acids acidic, neutral and basic amino acids (Sigma, St. Louis, Missouri, USA). Norleucine (250 nmol mL^{-1} in sodium citrate buffer, 0.2 M, pH 2.2) was also used as an internal standard.

3.2.14 Trypsin inhibitor activity

Trypsin inhibitor activity was assessed according to the method described by Stauffer (1990). The determination was based on the decrease in the hydrolysis rate of substrates (added bovine trypsin) caused by the inhibitor. The method involves extraction at pH 9 of the inhibitors, which were added with bovine trypsin. The activity of the remaining trypsin was measured with N-α-benzoyl-D, L-arginine p-nitroanilide

hydrochloride (BAPNA) under standard conditions. The amount of p-nitroaniline formed during 10 min incubation was measured spectrophotometrically and absorbance values in its presence and absence are used to calculate the number of trypsin inhibitor units (TIU) per milligram of sample.

3.3 Microscopic analysis

The microstructure of the seed was analyzed by three microscopic techniques.

3.3.1 Scanning Electron Microscope (SEM)

The surface morphology of the jicaro seed was scanned by a scanning electron microscope (SEM) at 50 mA (S-3700 Hitachi High Technologies, Tokyo, Japan). The samples were coated with an ultra-thin film of gold to make them conductive before analysis by 2 min two times. The scanning electron micrographs were obtained in the Research Center Microscopic Structures at the Costa Rica University.

3.3.2 Histological analysis of dehulled jicaro seeds

The first step was the fixing and then inclusion of the sample. The sample was initially set 1 hour under a moderate vacuum in a straight solution of 2 % paraformaldehyde, 1 % glutaraldehyde, 1% caffeine in a phosphate buffer (NaH_2PO_4 / Na_2HPO_4 0.1 M; pH 7.2). After one night at 4 °C the dehydration was carried out by passing successive butanol baths 50 ° for 1 h, 70° for 1 h). Following baths were made in microwave Histos 5 Milestone (70 °, 90 °, 100°, 100° / butanol; butanol 100%; butanol/resin and resin Technovit® 7100 or methyl methacrylate (MMA) Heraeus Kulzer, GmbH, Germany), for three hours in total. After a night in a bath of pure resin the inclusion of explants was carried out in plastic molds. The polymerization of the resin was carried out within 2 hours at room temperature. Thin sections (3.5µm) were obtained using a microtome (Leica RM 2255 GmbH, Nussloch, Germany). Sections were stained in oil red O for 10 min. Next sections were stained in Naphtol Blue–Black (NBB, sigma Aldrich) for 5 min. Sections were viewed and photographed using a microscope Leica Digital Microscope DM6000 (Wetzlar, Germany).

3.3.3 Confocal laser scanning microscopy

Jicaro seed was examined using confocal laser scanning microscopy CLSM (inverted microscope NIKON Eclipse-TE2000-C1si (NIKON, Champigny sur Marne, France). Thin slices of dehulled seeds, measuring approximately 5 mm × 5 mm × 3 mm

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LE NUMERO 1 MONDIAL DU MÉMOIRES

thick, were prepared from the freshly cut samples, using a scalpel. The protein network and neutral lipids were stained respectively with a mix of syto-9™ fluorescent dye (Kit LIVE/DEAD BacLight™ Molecular Probes, Invitrogen), prepared in water with a concentration of 24 µM and Nile red fluorescent dye (5H-benzo-R-phenoxazine-5-one, 9-diethylamino-; Sigma-Aldrich, St Louis, MO), prepared in propanediol at a concentration of 0.1 % w/v. These two solutions of fluorescent dyes were mixed just before the observation at a ratio of syto 9™/nile red 2:1. Then 10 µl of this mix were dropped on the slice and stored for diffusion for 15 min at room temperature. The dyed slices were observed with an oil immersion objective x100 magnification. Confocal observations were performed using an argon laser operating at 488 nm excitation wavelength (emission was detected between 500 and 530 nm) and a He–Ne laser operating at 543 nm wavelength excitation (emission was detected between 565 and 615 nm) (Sadat-Mekmene et al., 2013).

3.4 Color measurement

The sample color (jicaro seed and jicaro milk) was measured with the L*a*b* system, using a colorimeter Konica Minolta CR-400 (Sensing, INC., Japan), specified by the Commission Internationale de l'Eclairage (CIE). Tristimulus values give a three-dimensional value for the L*a*b* color space. The L-value represents light–dark spectrum with a range from 0 (black) to 100 (white). The a-value represents the green–red spectrum with a range from -60 (green) to +60 (red). The b-value represents blue–yellow spectrum with a range from -60 (blue) to +60 (yellow). Color measurements were performed in triplicate.

3.5 Analyses of volatile compounds

Two extraction techniques were selected for the analysis for the volatile compounds: Headspace Solid Phase Micro Extraction (HS-SPME) and Solvent-Assisted Flavor Evaporation (SAFE).

3.5.1 Headspace Solid Phase Micro Extraction (HS-SPME)

1.5 g of roasted seeds were placed and sealed in 10 mL headspace vials and extracted by HS-SPME (Lebrun et al., 2008). Extraction was carried out at 60 °C with 15 min incubation, 30 min trapping and shaking, using a polydimethylsiloxane / divinylbenzene fiber (PDMS/DVB 65 µm, SUPELCO, Bellefonte, PA, USA).

3.5.2 Solvent-assisted flavor evaporation (SAFE)

Unroasted, roasted, and dehulled roasted jicaro seeds were ground with liquid nitrogen in a grinder (Moulinex AR1105, France). 80 g of each sample were placed in a 500 mL Erlenmeyer, spiked with 24 µg internal standard of trans-2-octenal (Sigma-Aldrich, France). They were extracted twice with 150 mL of pentane–ether mixture (1:1 v/v) under stirring and a delicate flow of gaseous nitrogen for 30 min, and then filtered over anhydrous sodium sulfate. The volatile fraction of the filtrate was isolated by high-vacuum distillation (4.5 10^{-3} mbar) using SAFE at 45 °C. Distillate with volatile compounds was trapped in a 500 mL flask, and cooled with liquid nitrogen. Distillation lasted 120 min until high vacuum was reached. The solution was thawed and concentrated to about 500 µL in a Kuderna Danish glassware (KD) in a water bath at 45 °C. Extractions and analyses were conducted in triplicate

3.5.3 Gas Chromatography / Mass Spectrometry (GC/MS) of SAFE extracts

A tandem gas chromatograph 6890/MSD 5973N (Agilent Technologies, Palo Alto, USA) and a Gerstel autosampler MPS-2 was used. A non-polar capillary column DB-5 (5% diphenyl, 95% dimethyl siloxane), 30 m length, 0.250 mm I.D., 0.25 µm film thickness and a polar capillary column DB-Wax (polyethylene glycol) 30 m length, 0.25 mm I.D., 0.25 µm film thickness (both from J&W Scientific, Folsom, CA, USA) were used with a carrier gas hydrogen flow rate of 1.2 ml/min. 1 µl on-column injection at 45 °C was eluted with the following temperature program: 3 °C per min from 40 °C to 170 °C, then 10 °C per min up to 240 °C and held for 10 min. Mass spectrum were recorded in EI^+ mode at 70 eV within 40 to 350 Da. Analyzer and source temperatures were 150 °C and 250 °C respectively. Data were analyzed with Masshunter version B. 06.00 (Agilent Technologies, Palo Alto, USA). Peaks identification was performed by comparing their mass spectra with those of NIST 2011 (National Institute of Standard Technology) data base. Co-injection on both columns, of alkanes from C_8 to C_{20} (Sigma-Aldrich, St. Louis, MO, USA) was used to calculate Kovats retention indexes (RI) to compare them with those found in Flavornet, Pherobase and NIST websites.

Regarding SAFE extracts, the quantification of the whole volatile compounds was expressed as trans-2-octenal equivalent, considering the ratio of response coefficients equal to 1.

For the specific quantification of ethyl-2-methylbutyrate extracted both with SAFE and SPME methods, calibration curves were carried out with a pure standard (Sigma-Aldrich, (St. Louis, MO, USA)). The determination of response coefficients was performed, based on the extraction of 57 ions as quantifier ion and on the extraction of 102 and 85 ions as qualifier ions. For SAFE extract, a three-point calibration curve was performed, with a linear regression coefficient R^2 = 0.9988. For SPME/ODP extract, a three-point standard addition calibration curve was performed, with a linear regression coefficient R^2 = 0.9626. All the calibration curves were performed in triplicate.

3.5.4 Olfactory Detection Port (ODP) of SPME extracts

A panel of ten untrained panelists (five women and five men of various ages, from 28 to 55 years old) was recruited among the staff of the research unit at CIRAD, Montpellier, France. Two sniffing sessions of 35 min each, after which no odor was detected, were conducted on different days. The panelists sat comfortably with their nose placed in the nosepiece of ODP flushed with humidified air (100 ml/min) in order to avoid dryness of the nasal mucosa. The panelists were asked to give a qualitative perception and description of fragrances of jicaro seeds with their own words, while sniffing. Comments were automatically reported on the chromatogram as peak annotations.

GC/MS analyses of SPME extracts were carried out with the conditions described above, using the same DB-Wax column and an Olfactory Detector Port 3 (ODP3, GERSTEL GmbH & Co. KG). Injection: splitless mode for 30 s at 250 °C. Split ODP/MSD flow ratio 1/1, ODP port at 200 °C. Data were recorded with Gerstel ODP recorder ver 2.13.

The odor activity value (OAV) (Francis and Newton, 2005), which is the ratio between the concentration of an individual compound and the perception threshold found in the literature, was calculated for ethyl-2-methylbutyrate.

3.6 Proteomic analysis

The analysis of global protein composition was performed by the service Proteomics Laboratory Clodomiro Picado Institute, University of Costa Rica.

3.6.1 Quantification of protein

Jicaro seeds (10 g) were ground under liquid N_2 using a mortar and pestle. Frozen pulverized plant material (300 mg) was divided into two aliquots and extracted with 500 µL 0.1 M Tris-HCl buffer (pH 6.8). After centrifugation (10,000 × *g* for 5 min, 4 °C), the supernatants of both aliquots were combined and used for protein fractionation. Total protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Berkeley, CA, USA) with bovine serum albumin (BSA) standards.

3.6.2 Reverse phase HPLC Electrophoresis

The crude extract (1 mg in 200 µL) was fractionated using reverse-phase high-performance liquid chromatography on a C_{18} column 4.6 x 250 mm, 5 µm particle diameter (Teknokroma Barcelona, Spain) with an Agilent 1200 chromatograph monitored at 215 nm (Agilent, Santa Clara, CA, USA). The flow rate was 1 mL/min, and the elution was performed by applying a gradient from 0.1% trifluoroacetic acid (TFA) in water (solution A) to acetonitrile containing 0.1% TFA (solution B) as follows: 0% B for 5 min, 0 – 15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min, as previously described by Lomonte et al.,(2014). The fractions were collected manually and evaporated by vacuum centrifugation (Eppendorf Vacufuge, Germany) at 45 °C.

3.6.3 Electrophoresis

Once dried, the samples were reconstituted in water and mixed with reducing buffer for further separation by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% pre-cast gradient gels (Bio-Rad, Berkeley, CA, USA).

3.6.4 Mass spectrometry

Protein bands were stained with Coomassie G-250 and digitally recorded in a Chemidoc® imaging device (Bio-Rad). Then, protein bands were excised from the gels, reduced with dithiothreitol, alkylated with iodoacetamide, and digested with sequencing-grade bovine trypsin for 16 h in an automated digester (Progest, Digilab). Resulting peptides were mixed with an equal volume of a saturated solution of α-cyano-hydroxycinnamic acid in 50% acetonitrile with 0.1% TFA, and 1 µL of each peptide mixture was spotted onto an Opti-TOF 384-well plate, dried, and analyzed in positive-

reflector mode using MALDI-TOF-TOF mass spectrometry model 4800 Proteomics Analyzer Plus instrument (Applied Biosystems, Foster City, CA, USA.). TOF spectra were acquired using 1500 shots and a laser intensity of 3000. The ten most intense precursor ions were automatically selected, and their TOF/TOF fragmentation spectra were acquired using 500 shots at a laser intensity of 3900. External calibration in each run was performed with CalMix® standards (ABSciex, Framingham, Massachusetts, USA) spotted onto the same plate. For protein identification, resulting spectra were searched against UniProt/SwissProt databases (general and Viridiplantae) using ProteinPilot® v.4 and Paragon® algorithm (ABSciex, Framingham, Massachusetts, USA) (≥ 95% confidence), and spectra were submitted to MASCOT public server (Matrix Science). Peptide sequence spectra with lower confidence scores were manually interpreted and searched using BLAST (http://blast.ncbi.nlm.nih.gov) for protein similarity and family assignment. A few tryptic digest samples were analyzed by direct infusion in nano-ESI-MS/MS using a QTrap3200 instrument (Applied Biosystems, Foster City, CA, USA) operated in positive mode. Doubly or triply charged peptide ions were fragmented by collision-induced dissociation, and the resulting MS/MS spectra were interpreted by manual *de novo* sequencing.

3.7 Roasting jicaro seeds

The roasting of jicaro seeds were conducted in an electrical Probat roaster BRZ2, shown in **Figure 3.1**. The roaster consists of two rotating drums (500 length x 670width x 560 mm thickness) that include 2 attached thermometers 0 – 300 ºC, to indicate the roasting temperatures, each with thermos-sensors and capillaries, 2 cooling sieves, 2 complete electrical heating device (drums). Each drum has a diameter of 3.8 cm and electric heating of 850 W, including chaff cyclone, volume flow fan 160 m^3/h, exhaust air volume flow 50 m^3/h.

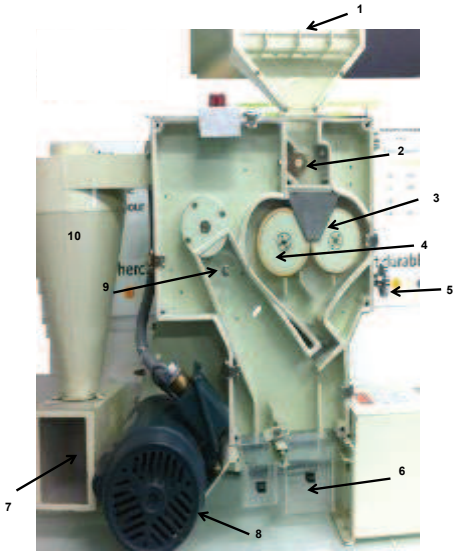
Raw jicaro seeds (**Figure 3.3**, left) were placed in portions of 30 grams into the cylinder of 80 – 100 g capacity per batch. Seeds were roasted in permanent rotation. The temperature was adjusted by regulating the heating current and the flow of air through the drum. Different combinations of temperature and roasting time (120, 140, and 160 °C and 80, 100, 120, 140, 160 and 180 s respectively) were tested with the goal of: a) obtaining more than 80% of open seeds (see **Figure 3.3**, middle), b) preserving the typical aroma of jicaro and c) keeping the kernel color with a maximum variation in

brightness of 5% of the initial value (Li = 79). Permanent rotation of the cylinder enabled uniform roasting conditions. Air velocity in the roaster was measured with a propeller anemometer Voltcraft BL-30 AN, and the temperature was registered using K-type thermocouples.

Figure 3.1. *Coffee roaster used for roasting jicaro seeds*

3.8 Dehulling jicaro seeds

After roasting, the seeds were tempered with different combinations of moisture (7.5, 10, 12.5, and 15 %) and conditioning time (1, 2, and 4 hours). Before dehulling, seeds were placed in bottles and stirred occasionally. After the tempering time, roasted jicaro seeds were dehulled with a laboratory Satake dehuller (Model THU 35B, Hiroshima, Japan) (see **Figure 3.2**), usually used for rice and adapted to this small and flat seed. The dehulling was performed during the passage of the seeds between two rubber rolls, rotating in opposite directions at different speeds (1; 1.25), with a space between rolls adjustable according to the seed size (here, 2 mm thickness). This differential speed causes a friction phenomenon at the surface of the seed coat, which is removed and then driven into a cyclone (air speed 0.86 m/s) by a ventilation system. The dehulled seed (**Figure 3.3**, right) is recovered in buckets. For jicaro seeds, the feeding was carried out in batches of 10 g, with a feed rate controlled by using a mill feeder (3170 Perten, Hamburg, Germany).



1. Feed hopper
2. Feed roller
3. Fast rubber roller
4. Slow rubber roller
5. Rubber roller adjusting handle
6. Outlet of hulled seeds
7. Outlet of seed coat
8. Blower
9. Wind adjuster
10. Cyclone

Figure 3.2. *Satake dehuller*

One metric was used to assess the effectiveness of the dehulling: the dehulling efficiency. The *dehulling efficiency,* D_{ef}, represents the amount of dehulled seed which is obtained from the roasted seeds. It is defined by the following expression

$$D_{ef} = \frac{w_{\text{dehulled}}}{w_{\text{in}} \times R_{\text{coty}}} \times 100$$

where w_{dehulled} is the weight of the dehulled seeds, w_{in} the weight of the roasted seeds introduced into the machine and $R_{\text{coty}} = w_{\text{coty}}/w_{\text{seed}}$ the ratio between the weight of the cotyledon and the total weight of the seed.

Figure 3.3. *Jicaro seeds: raw (left), roasted (middle) and dehulled (right)*

3.9 Process developed for making jicaro milk

3.9.1 Raw materials

Jicaro milk was made from the dehulled jicaro seeds.

Jicaro seeds preparation: seeds, containing the seed coat (hull) and cotyledon were weighed and then were conditioned with water.

Tempering: Jicaro seeds were tempered at various moisture contents, from 6 to 15 % moisture. 1 kg of whole seeds, was added with distilled water using a spray. After addition of water, jicaro seeds were placed into a closed container for 24 hours and agitated frequently for distributing water regularly on the surface of the seed. The amount of water added was determined according to the following mass balance equation:

$$Water\ (\mathrm{g}) = w \times \frac{m_d - m_i}{100 - m_d}$$

where w is the weight of jicaro seeds (in g); m_d the desired moisture content (in %); and m_i the initial moisture content (in %).

Roasting: Jicaro seeds (200 g by batch) were roasted in fluidized bed (Neuhaus Neotec, Reinbek, Germany) at 220°C during 60 s. The seeds were judged to be roasted when most of them started to puff, with a clear puffing sound, and a visible crack in the seed coat. After roasting, the seeds were cooled at room temperature.

Tempering: Roasted seeds were conditioned at various moisture contents (7.5, 10, 12.5, and 15 %) using a spray and stirred. The amount of water added was determined according the mass balance equation described above.

Dehulling: Conditioned roasted seeds were dehulled by the Satake dehuller (Model THU 35 B, Japan, described in Section 2.8) (see **Figure 3.2**) to remove the seed coat and get the dehulled seeds.

Soaking: 100 g of dehulled seeds were soaked into a water bath. The soaking water was added at three different temperatures (60 °C, 70 °C and 80 °C) and three soaking time (1, 2 and 3 hours).

Draining: After soaking, dehulled seeds were drained in a stainless-steel strainer sieve during 20 minutes.

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Grinding: Dehulled seeds were reweighed to determine the amount of water absorbed by them. They were added with twice their wet weight (corresponding to a ratio of 1:3) before crushing in a heating blender (Moulinex Soup & Co LM9051B1, French) for two minutes at the maximum speed.

Filtration: The slurry was filtered in a muslin cloth (214 µm of mesh opening and 204 µm of thickness) while applying manual pressure to extract the maximum of jicaro milk and separate it from the jicaro cake.

Bottling: the jicaro milk was placed in glass bottles (250 mL) and stored at the freezer at 5ºC.

3.9.2 Homogenization

Homogenization was carried out on a SPX-APV-Gaulin high pressure homogenizer at laboratory-scale. Three pressures were tested: 23, 50 and 75 MPa at a flow rate of 23 L/h.

3.9.3 Physico-chemical analysis of the jicaro milk

3.9.3.1 Suspension stability

Suspension stability was evaluated after storage of jicaro milk for 24 hours at 5°C. For visual stability, 200 mL of jicaro milk were placed in a graduated glass bottle and observed for separation into layers. When a line of demarcation was observed, the ratio of height of sediment to total height of jicaro milk was calculated and defined as the visual stability index. If no separation was observed, the visual stability is equal to 1 (where 1.0 = maximum stability) (McClements, 2007).

3.9.3.2 Particle size

Particle size distribution was determined by laser light scattering using a Mastersizer 3000 (Malvern Instruments Ltd, Worcestershire, UK) and log-spaced array detector in a range between 0.01 µm and 3500 µm. This method involved loading the sample into a dispersant unit (Hydro MV, Malvern Instruments Ltd, UK) filled with water as the suspension medium under controlled conditions (obscuration of 39 % and stirring of 1500 rpm) (Dahdouh et al., 2016). Before loading, each sample was divided into several approximately equal quantities, and consecutive 4-second measurements. The dispersed sample passed though the measurement area of the optical bench, where a laser beam illuminates the particles. The detector then accurately measured the intensity

of light scattered by the particles within the sample for both red and blue light wavelengths and over a wide range of angles. This data were analyzed to calculate the size of the particles that created the scattering patter. The value 1.46 was attributed to the refractive index of cloud and dispersant phase (water).

These settings were selected because under these conditions, the samples were well dispersed into water. The samples were analyzed immediately after preparation in triplicate.

3.9.3.3 Rheological characterization.

Rheological measurements were performed using a Physica MCR301 Rheometer (Anton Paar GmbH, Austria) equipped with a Couette flow measuring cell (Ref. DG27/T2000/SS), according to the procedure Dahdouh et al., (2015).

The samples were placed between two rigidly interconnected at fixed coaxial surfaces (stator) with a cylinder symmetry double – gap (26.7 mm diameter and 1 mm gap). All measurements were conducted at 20 ± 0.05 °C using a Peltier system and a fluid circulator Viscotherm VT 2 controlled directly from the Physica MCR. Jicaro milk flow behavior was determined by increasing logarithmic ramp (recording 5 points/decade) of the applied share rate ($\dot{\gamma}$, from 0.1 s^{-1} to 5000 s^{-1}); experimental results were analyzed through the representation of the apparent. All samples were performed in triplicate.

3.9.3.4 Crude protein

Jicaro milk (in samples of 10 mL) was analyzed by Kjeldahl's method as described in Section 3.2.2.

3.9.3.5 Crude fat

Jicaro milk was divided in samples of 200 mL, which were lyophilized independently using a laboratory bench top freeze dryer model P6L85V (CRYONEXT, France). Samples' temperature was monitored between -20 ºC and 20 ºC according to tray temperature. Freeze-drying was stopped when the water was completely removed. Then 0.5 g of sample were analyzed by solvent extraction (ethyl ether) using the soxtec system as described in Section 3.2.3.

3.9.3.6 Ash

After freeze-drying, 0.5 g of sample were analyzed as described in Section 3.2.4.

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LE NUMERO 1 MONDIAL DU MÉMOIRES

3.9.3.7 Color

Each jicaro milk sample (10 mL) was poured into a sample cup and placed over the instrument port for measurement. The color determination was carried out as described in Section 3.4.

3.9.3.8 Tocopherols

The extraction procedure was adapted as described previously (Rossetti et al., 2010). Each sample (0.5 mL for jicaro milk or 0.5 g for cake) was placed in a glass tube, with 2 mL of pyrogallol 1% in ethanol and mixed with vortex for 1 minute. Then 1.5 mL of 12 N KOH was added to each tube, and the samples were saponified for 30 min at 70 °C and were extracted twice with 5 mL n-hexane. The chromatographic conditions are described in Section 3.2.11.

3.9.3.9 Volatile compounds

Volatile compounds of jicaro milk were analyzed by Headspace Solid Phase Micro Extraction (HS-SPME) as described in Section 3.5.1.

3.9.3.10 Analysis of milk microscopy

Jicaro milk was analyzed by confocal laser scanning microscopy as described in Section 3.3.3.

3.10 Nutritional Intervention Study on the jicaro milk

3.10.1 Determination of the glycemic index (GI)

The clinical study and the preparation of the test meals were performed at the National Center for Food Science and Technology in San José, Costa Rica, called by its Spanish acronym CITA. The clinical trial was carried out with 12 volunteers (six men and six women). The experimental protocol was approved by the Scientific Ethics Committee of the University of Costa Rica (UCR).

The volunteers were recruited from the CITA and had to meet the following criteria: 1) no smoking, 2) no family history of diabetes, 3) apparently healthy, 4) signed informed consent. They were oriented about not drinking alcohol, not eating in excessive amounts, no drugs and no strenuous exercise the night before the study.

Three foods were assessed by the volunteers: the jicaro beverage (240 mL), soy milk with natural vanilla flavor (SILK SOY MILK) (472 mL) and white bread (54.70 g)

which is the standard food. The carbohydrate intake of all tested foods was 25 g of available carbohydrate.

The intake was performed after a fasting period of 8 h. Each volunteer took a blood sample by pricking the finger pads of the non-dominant hand. The blood glucose levels were measured at intervals of 30, 60, 90 and 120 minutes after the start of the meal. Glycemic responses were calculated for each subject according to FAO/WHO standards (FAO/WHO, 1998) and analyzed at the Laboratory of biochemistry at the school of medicine, UCR. The Glycemic responses were determined by means using an Accu-Chek blood glucose meter (Roche Diagnostics, S.L., Colombia).

Glycemic index (GI) was determined for jicaro beverage and soy milk using white bread as reference and calculated as follows:

$$\text{Glycemic index} = \frac{\text{Glucose response to test food}}{\text{Glucose response to standard food}} \times 100$$

3.10.2 Metabolomics study of urine after consumption

3.10.2.1 Study design

The metabolomics analysis in humane urine samples was performed by collecting urine fractions of 0-6, 6-12 and 12-24 hours before and after administering a dose of 240 mL of jicaro milk. It was a short-term exposure study (7 day) and the recovery of urine was carried out for 24 hours after the day of the last ingestion.

Eight volunteers of male gender took part in a screening session. Anthropometrics measure (height, weights, and body mass index (BMI)), blood pressure and biochemical parameters (creatinine, urea, transaminases and gammaglutamyltransferase) were measured for each volunteer before and after of the study.

Inclusion and exclusion criteria were established for the selection of volunteers.

The following **inclusion criteria** were taken into account:

- Male gender.
- Age between 18 and 30 years.
- People who like or tolerate the organoleptic properties of jicaro.
- Body mass index (I.M.C in kg/m^2) 19 – 27

- Declaring survey pre-selection "in good health and no history of serious chronic and infectious diseases."
- Not having family or individual history of hematological abnormalities.
- Not using food supplements or vitamins within 6 weeks before the start of the study.
- Not taking drugs in the 15 days before the beginning of the study.
- Analytical results of renal and hepatic function within the normal range.

Exclusion criteria:

- Previous history of alcohol abuse, or drug snuff.
- Consumer of more than 4 cups of coffee or tea per day.
- Background of having undergone surgery (abdominal, thoracic, etc.) in the six months preceding the start of the study.
- History and / or presence of any gastrointestinal renal disease, or liver.
- History of allergy, idiosyncrasy or hypersensitivity to jicaro.
- Smoker.
- High performance athletes.

The study design was established so that each subject was its own control. Urine samples were collected in container of polyethylene at the laboratory of Costa Rica university.

Food intake was controlled using a list of forbidden foods for the days of the study. It was enforced to limit the possible taking of foods containing jicaro seeds. During three days (1, 2, and 7) a standard diet for the breakfast, lunch and dinner was supplied for all volunteers.

Each volunteer was requested to collect his own urine for 24 hours on the first day and the seventh day of the study. On the first and seventh day, the urine collection was divided into three periods: 8 am to 2 pm (sample named urine 0 - 6 h); 2 pm to 8 pm (sample named urine 6 -12 h); and 8 pm to 8 am on next day (sample named urine 12 – 24 h). On the first day of the study they were asked to take 420 mL of water with sugar. On the second day, they were asked to take 420 mL of jicaro milk.

For days 3, 4, 5, and 6 the volunteers were asked to take 210 mL of jicaro milk every day at 8 am, but no urine sample was collected. On the seventh day of the study

they were asked to collect their own urine for 24h (sample named total urine 24h). **Table 3.1** shows the study design.

Table 3.1. Schema of urine sample collection

Days	**1**			**2**			**3**	**4**	**5**	**6**	**7**
Diet	**Standard**			**Standard**			**No standard**				**Standard**
	+ 420 mL water with sugar			**+ 420 mL of jicaro milk**			**+ 210 mL of jicaro milk**				
Urine collection	0-6h	0-12h	12-24h	0-6h	0-12h	12-24h	No urine collection				0-24h
Sample	Control			After consumption of jicaro milk							

The urine samples were stored in refrigeration for 12 hours. After this time, the urines were separated into aliquots and frozen at -70 °C until analysis.

3.10.2.2 Analysis by mass spectrometry

Sample preparation

Urine samples were defrosted at room temperature. Subsequently, they were centrifuged for 10 minutes at 13000 rpm. 500 µl of the supernatant was mixed with an equal volume of water MilliQ quality. The samples were vortexed for 30 seconds and then placed on the vials rack for the automatic injection into UPLC-MS equipment.

Analysis

Identification of secondary metabolites was performed using an Ultra High-Pressure Liquid Chromatography- Diode-Array Detector/ Electrospray ionization - Quadrupole time-of-flight tandem mass spectrometry-Mass Detector) (Waters SYNAPT G1, Waters Corp., Milford, MA, U.S.A.).

A column Acquity UPLC-C_{18} (1.7 µm 2.1mm x150mm) was used. A volume of 6 µl of each diluted urine sample (50% water / 50% urine, v / v) was injected. In addition, a mixture of known standards was used, which contained phenylalanine, tryptophan, creatinine and amino anthracene, at a concentration of 5 µg / ml. Besides, a pool will all urine collected for the same test was made to be used as quality control.

Samples were randomly injected in groups of 10 samples and inserted between a solution of methanol, a mixture of standard and the urine pool of all samples. Mobile

phase components were water with 0.1% v / v of acid formic (solvent A) and another part of acetonitrile with 0.1% v / v of acid formic (solvent B). The flow rate was 400 µl / min. The column was eluted at a flow rate of 400 µL / min with a gradient of 0 % B increasing from 0 to 10% over 7 minutes, followed by an increase from 10 to 95% over 7 – 22 minutes. The mobile phase was then returned to 100% A at 22.1 minutes for 4 minutes of re-equilibration.

The mass spectrometry data were collected in continuous full – scan mode with a mass to-change ratio (m / z) of 70 to 1000, from 0 to 26 minutes in the positive mode. The source temperature was set to 120° C with a cone gas flow of 40 L/h, a desolvation temperature of 330 °C and a nebulization gas flow of 650 L/h. The capillary voltage was set to 3000 V, and the sample cone voltage was set to 30V.

3.10.2.3 Data processing

Data processing. The raw data collected in a continuous mode were centered using the lock mass spray of leucine-enkephalin and transformed to NetCDF format using Databridge application (MassLynx SCN639 v 4.1). Chromatograms were deconvoluted using the software package MZmine 2.3 (http://mzmine.sourceforge.net/), resulting in a 3-dimensional data set with retention time, *m/z* and intensity for each sample.

Statistics. Datasets were analyzed using both univariate and multivariate data analysis. Data were normalized for urine volume prior to any statistical analysis. The data were processed using a univariate two-way analysis of variance (ANOVA). The ANOVA model included the diet effect (water versus Jicaro milk) and the subject effect. The p-value was calculated, and p-values below 0.05 were considered statistically significant. Unsupervised Principal component analysis (PCA) was performed first followed by supervised partial least-squares discriminant analysis (PLS-DA) with orthogonal signal correction (OSC, 2 components, sum of square remaining = 62.7 % with data log transformed and filtered by Pareto scaling) (Xi et al., 2014) to assess linear combinations of ions responsible for the differences between groups using SIMCA (version 12.0, Umetrics AB, Umea, Sweden). Statistical validity of the model was assessed by three complementary tests according to good statistical practice (Eriksson and Lindström, 2006; Xue et al., 2014). First, the prediction power of the model Q^2X parameter was assessed by the 7-fold cross validation method. Second, was carried out the response permutation testing (with n=100) to check that PLS

components could not lead to a correct classification by chance and evaluate data overfitting. The result of this test is displayed on a validation plot, which shows the correlation coefficients R^2 and Q^2 for permuted samples and the original non-permuted sample X at abscissa 1 corresponding to ordinate Q^2X and R^2X. Two regression lines of the different R^2 and Q^2 values are traced and ordinate at abscissa 0 are reported. For validation of the model, the regression line of Q^2 must have a negative intercept (Eriksson and Lindström, 2006). Finally, the significance of the model was assessed using a cross-validated analysis of variance (CV-ANOVA) by the SIMCA software. The Variable Importance Projection (VIP) values were obtained as indicators of the importance of each ion in the discrimination.

Metabolite identification. All the significant ions (p-value <0.05 and VIP>1) that most contributed to the separation were clustered according to their retention time Then, in each selected cluster, ions were classified according to p-value in increasing order. Clusters were then classified in descending order according to the p-value of the first ion of each cluster. In these conditions, clusters are assumed to correspond to one specific compound that included some adduct, fragment and isotope ions that had been created during ionization in the source. Then, within each cluster, possible adducts and isotopes were recognized in order to tentatively identify the parent ion $[M+H]^+$. The parent ion, or other interesting adducts ions, were fragmented by MSMS following the same chromatographic method described for urine profiling using different collision energies of 15, 20, 25, 30, 40 eV and a ramp from 10 to 40 eV. Finally, parent ions were annotated only if differences between the observed accurate mass (m/z) and the theoretical mass of the proposed compound was inferior to 5 ppm and if the MSMS fragmentation pattern corresponded with those of standard, data published in the literature or reported in a database. When it was not possible to comply with these requirements, the metabolite was catalogued as "Unknown". The web-based databases consulted were the METLIN Metabolite Database (www.metlin.scripps.edu/index.php) (Smith et al., 2005). Human Metabolome Database (HMDB; www.hmdb.ca) (Wishart et al., 2013), Massbank (www.massbank.jp) (Horai et al., 2010) and *ReSpect* (*http://spectra.psc.riken.jp*) (Sawada et al., 2012).

Chapter 4

Results

The results of the thesis are presented in the form of scientific publications. The first three works are presented as accepted / submitted for publication, while the study on the jicaro milk contains some preliminary results.

In Section 4.1 the results of the physico-chemical characterization of the jicaro seed are shown. Macronutrients, micronutrient, proteomic profile, functional compounds and antinutritional factors were analyzed by different methods and techniques. No chemical or thermal treatment was applied in the preparation of the samples to preserve the integrity of their composition. The main chemical composition proved to be a seed of high nutritional value comparable to soybean but with the exception that in the jicaro seed the antinutritional factors were found in insignificant amounts. An interesting result was the proteomic profile, which revealed the existence of protein related to embryogenesis, heat shock proteins, which may explain the exceptional resistance of jicaro to extreme climate from dry regions.

In Section 4.2 the results of the effect of a mild roasting (140 ºC for 140 s) in both the physico-chemical composition and the volatile compounds of jicaro seeds are presented. This is the first time the effects of a mild roasting is studied and the work showed that at those conditions the Maillard reaction was also taking place. 27 volatile compounds were identified and quantified by Chromatography/Mass Spectrometry

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(GC/MS). Among them, the ethyl-2-methylbutyrate was recognized as the key jicaro by means of a gas chromatography olfactometry (GC–O) analysis. As a result of roasting its concentration increased. Pyrazines appeared as well, contributing to the pleasant scent of the roasted jicaro. Regarding the physico-chemical composition, a slight impact on polyphenol, fructose and free amino acid contents was observed. Besides, the roasting caused changes in the microstructure which resulted in the coalescence of the lipid bodies.

In Section 4.3 the results of the optimization of roasting in combination with the tempering for the dehulling of the jicaro seed are presented. Opened seeds yield, moisture content, and color were the parameters taken into account for the optimization. Kinetic modeling showed that the dehydration of the jicaro seed presents a first order kinetics in the studied range of temperatures (120 to 160 ºC). Dehydration and popping are linked and it was possible to attain 90% of open seeds while keeping the lightness of the roasted cotyledons. The key aromas of the jicaro seeds were maintained as well after roasting. The tempering of the roasted jicaro seeds allowed the efficient dehulling of the seeds. Under the best conditions it was possible to extract 75% of the cotyledon material.

In Section 4.4 the preliminary results on the elaboration of a milk from the jicaro cotyledons are described. A process flowsheet was proposed, including soaking, grinding and filtering phases. The results showed that the obtained milk exhibited similar nutritional properties as other vegetal milks such as the ones made from soybean and almond. The isolation of the cotyledons to be used as the raw material allowed the obtaining of a final product with a light color, which was a constraint to make it competitive with other vegetal milks of the market. The resulting milk presented some stability problems; this is why an additional homogenization phase was also explored, although the results were preliminary. A clinical study also showed that the jicaro milk has a low glycemic index, adding to its healthy properties.

4.1 Physico-chemical characterization of jicaro seeds (*Crescentia alata* H.B.K.): a novel protein and oleaginous seed

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Abstract

Jicaro (Crescentia alata) is a tropical tree native to Mesoamerica well adapted to severe drought conditions. The seeds of the fruits were analyzed for protein, fatty acid, dietary fiber, phytate, polyphenol, tannin, tocopherol, sugar, mineral, amino acid and trypsin inhibitor contents. The jicaro cotyledons contained 43.6 ± 1.15 g protein/100 g and 38.0 ± 0.20 g fat/100 g (d.w.), which is comparable to most protein-rich and oleaginous seeds. Among the lipids, 77.6% were unsaturated fatty acids, particularly oleic acid, and essential amino acids represented 16.0 ± 0.9% (d.w.) of the protein fraction, which is similar to soybean amino acid contents. A proteomic analysis and SDS-PAGE electrophoresis revealed that the proteins are mostly of low molecular weight (~10 kDa), and the storage protein 2S albumin dominated. Jicaro seed trypsin inhibitory activity was low (0.1 trypsin inhibitor units TIU/mg), which enhances the digestibility of its proteins. The jicaro seed cotyledon represents an autochthonous and high-quality food source.

Keywords

Food analysis, Food composition, *Crescentia alata,* Nutritional quality, Jicaro seeds, Biodiverse food, Oleaginous seed, Oil profile, Proteomic.

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Highlights

- Jicaro seeds contain high protein and lipid contents similar to soybean seeds
- The nutritional quality of the protein and lipid fractions is high
- Most jicaro seed proteins have a low molecular weight
- No anti-nutritional factors were observed
- Jicaro seeds could be considered as a highly nutritional food for dry areas

4.1.1 Introduction

Jicaro (*Crescentia alata),* which belongs to the family Bignoniaceae, is an ancient tree of American tropics (Vázquez-Yanes et al., 1999). Jicaro fruit has a calabash shape, and it was widely used during pre-Columbian era not only as a staple food but also as food utensils and containers. Jicaro fruit has a lignified pericarp that contains a sweet pulp surrounding many seeds. Jicaro fruit seeds, which represent 5% of the whole fruit, are dark brown, flat and heart shaped with a pleasant characteristic odor. Little research concerning jicaro exists, and few studies have attempted to characterize the composition of jicaro seed. Several decades ago, oil and fatty acid composition of jicaro seed was partially determined with respect to its main fatty acids (61.8% oleic, 15.0% linoleic) (Lewy, 1960). A more recent work performed on Guatemalan fruits showed that the seeds contained 38% fat and 26% protein (Figueroa Madrid and Bressani, 2000). These preliminary data were nonetheless of very high importance, as even in silvopastoral systems, with an average of 350 trees per hectare, each tree produces 750 fruits per year corresponding to 52 kg of fresh seeds (Carballo et al., 2005), which represents a high potential for commercial production. Additionally, the tree is well adapted to Pacific region of Central America, where severe droughts can occur, and thanks to deep root system, jicaro can absorb nutrients and water from savanna soils (Bucheli et al., 2013). Jicaro is one of the only trees to remain green year-round in the dry regions of Central America.

Jicaro seed was an important staple food in some pre-Columbian communities (Staller, 2010), and it is still widely consumed in Mesoamerica, especially in El Salvador and Nicaragua, as a very popular beverage called “horchata”. Nonetheless, because of real value of this seed remains unknown, consumption is decreasing and preservation of jicaro trees in silvopastoral systems must be strengthened. In the context of adaptation to climate change, a renewed interest in sustainable and biodiverse food

sources with high nutritional and functional potential, has led to the rise of new market opportunities. For this reason, the objective of the present study was to describe the physico-chemical characteristics of jicaro seeds and their main nutritional properties.

4.1.2 Materials and methods

4.1.2.1 Raw materials

Jicaro seeds (*Crescentia alata*) were obtained from the Caserio Los Zarzarles (latitude: 12.66°N, longitude: -86.44°W, altitude: 115.7 m) in the municipality of León, from Pacific region of Nicaragua in November 2013 and 2014. The seeds were prepared following a traditional process by seeds handler. Mature fruits were halved, and the seed-containing pulp was placed in polystyrene bags to ferment for three days. The seeds were then separated and washed before being sun dried. A total of 100 kg of dried seeds were collected from different jicaro fields, gathered and mixed at the Universidad Nacional Autónoma de Nicaragua (UNAN) of the city of Leon in the Pacific region. Foreign material was removed. The seeds were washed again and sun dried to obtain a unique homogenized batch, representative of the overall production. All analyses were performed on samples of this representative batch.

Cotyledons and seed coats were separated manually from jicaro seeds. Each seed part was ground separately into a powder by using a PREP'LINE knife blender (SEB, Ecully, France) before analysis. Commercial hulle yellow soybean (Markal, Saint-Marcel-lès-Valence, France) were bought in a supermarket in Montpellier, France and were analyzed as comparison witness.

4.1.2.2 Chemical analysis

Jicaro seed, cotyledon and seed coat samples were analyzed in triplicate. Dry matter content of 5 g sample was determined according to PR NF ISO 6496 standard using differential weighing after oven drying at 103°C to reach a constant weight.

Micro-Kjeldahl method was used according to the Official Method 950.48 (AOAC, 1995) to determine total protein content (N x 6.25) with an automatic Foss analytical AB Kjeltec™ 8400 apparatus (Foss, Höganäs, Sweden). Each sample was previously mineralized with concentrated sulfuric acid and a mixture of 0.15 g of $CuSO_4 \bullet 5H_2O$, 5.0 g K_2SO_4, and 0.15 g titanium dioxide (Thompson Capper Ltd., Hardwick, UK) (Venkatachalam and Sathe, 2006).

Fat content was determined by using an ASE™ 350 accelerated solvent extractor (DIONEX Corp., Sunnyvale, CA, USA). A 1 g sample was placed in the stainless steel extraction cell in the presence of 0.5 g sand. The extraction solvent was pure petroleum ether at 60°C and 100 bar. Flush was set to 100%, number of cycles to 5 with a static time of 7 min, and purge duration was 90 sec. The extraction solvent containing lipids was collected in a 50 mL flask that had been previously dried and weighed. Flasks were weighed again after total evaporation of the solvent. Lipid content was expressed as g fat per 100 g sample (dry weight basis).

4.1.2.3 2.3. Soluble sugars

Sugar analysis was performed on the lipid-free meal remaining in the extraction cell. A 80% ethanol solution (v/v) was used as the solvent at 60°C and 100 bar. Extracts of recovered sugars were diluted 25-fold with deionized water and filtered to 0.45 µm before injection of 10 µL into the chromatographe. Soluble sugars were separated using high performance ionic chromatography (HPIC) with a DX600 system equipped with a Carbopac MA-1 column (250 x 4 mm), a Carbopac MA-1 guard column (25 x 4 mm) and a Dionex ED50 pulsed amperometric detector (PAD) (DIONEX Corp., Sunnyvale, CA, USA). Chromatographic conditions were described by Valente et al. (2013).

The determination of α-galactosides (raffinose, stachyose and verbascose) was performed according to a procedure described by Muzquiz et al. (1999). A sample containing phenyl α-D-glucoside (100 µg) as an internal standard and 48% aqueous ethanol was added. Extraction was performed using a sonication for 60 min, followed by centrifugation at 700×g for 10 min. Combined supernatants were heated at 85°C under reflux for 30 min, cooled and centrifuged at 700×g for 5 min. The supernatant was evaporated to dryness. The residue was dissolved in water, and an aliquot was transferred into a glass-stoppered test tube. Acetonitrile (1.0 mL) was then added with shaking, and the mixture was stored overnight at 4°C. The sample was injected into an HPLC DX600 system (DIONEX Corp., Sunnyvale, CA, USA).

4.1.2.4 Fiber content

Neutral detergent fiber was prepared from 1 g sample according to the method of Van Soest et al.,(1973) ,which sequentially yields neutral detergent fibers (NDF), acid detergent fibers (ADF) and acid detergent lignins (ADL). For NDF, a neutral detergent solution (sodium lauryl sulfate, USP-grade and ethylenediaminetetraacetic acid, EDTA; pH 7) at boiling temperatures with a 0.2 mL of heat-stable α-amylase Termamyl®

(Laboratoires HUMEAU, La Chapelle-sur-Erdre, France) was used to dissolve the easily digested pectins and cell contents (proteins, starch sugars, and lipids), leaving a fibrous residue NDF (cellulose, hemicellulose and lignin). For ADF, 100 mL an acid detergent solution (20 g cetyl trimethylammonium bromide and 0.5 M H_2SO_4) were used to dissolve hemicellulose and minerals. Finally, for ADL, a 3 h digestion was performed with 72% H_2SO_4 at the crucible halfway up. Waste mineralization was performed at 550 °C for 4 h. The results are reported on a dry matter basis, as cellulose (ADF-ADL), hemicellulose (NDF-ADF) and lignin (ADL).

4.1.2.5 Mineral composition

Mineralization (500 °C) of the sample in an ash furnace (Thermo Scientific™ Thermolyne™ 6000 series 408, Waltham, Massachusetts, USA) was performed prior to the analysis of P, K, Na, Ca, and Mg and similarly for trace elements (Fe, Mn, Cu and Zn) until the ashes were cleared. Ash were then digested with hot concentrate hydrochloric acid until the destruction of organic matter, as described by Pinta, (1973) methodology. Mineral contents were performed by inductively coupled plasma atomic emission spectroscopy ICP - AES) (Varian Vista-Pro, Palo Alto, CA, USA) and quantified against standard solutions of know concentrations. Total mineral content was defined as the sum of all the minerals analyzed.

4.1.2.6 Phytate determination

Phytate content were measure as describe by Sekiguchi et al., (2000). Jicaro seeds (0.1 g) were placed in 2 mL 0.5 M HCl overnight at room temperature with constant stirring. After centrifugation (10 min, 10,000×g, 10 °C), the supernatant was recovered and then diluted in 0.2 M borax buffer (pH 8.0). Phytate content (inositol hexakisphosphate, IP6) was measured using HPIC with a DX600 system, equipped with an ATC-1 trap column, an AG11 guard column and an AS11 column, detection was performed with a conductivity cell connected to an ED50 detector after removing anions on an ASRS 300 (DIONEX Corp., Sunnyvale, CA, USA). The injected volume was 10 µL. Phytate elution was performed using a basic gradient of 200 mM NaOH. The gradient was linear from 30 to 80 mM NaOH for 8 min, before returning to and holding initial conditions for 8 min to re-stabilize the system. Data were analyzed with Chromeleon 6.0 software (DIONEX Corp., Sunnyvale, CA, USA). Calibration was done by using an external IP6 standard.

4.1.2.7 Total phenolic content

Total phenolic content was evaluated at 760 nm with the Folin-Ciocalteu reagent as described by Singleton, Orthofer & Lamuela-Raventos (1999). The lipid fraction was removed with hexane, and polyphenol extraction was performed on a 0.5 g sample added with 10 mL of acetone/water/formic acid (70/29/1, v/v/v). The results, in triplicate, are expressed as mg of gallic acid equivalents/100 g on a dry weight basis (mg GAE/100 g).

4.1.2.8 Tannin determination

Tannins were determined according to the ISO AFNOR NF V03-75 standard. They were extracted from a 1 g sample with dimethylformamide. After centrifugation (10 min, 10,000×g) and addition of iron (III) ammonium citrate, the absorbance of the supernatant was measured using spectrophotometry at 525 nm. The results are expressed as mg of tannic acid equivalents/100 g on a dry weight basis.

4.1.2.9 Tocopherol analysis

Tocopherols were extracted by using a method described by Taylor et al., (1976) and modified by (Deiana et al., 2002). A 0.5 g oil sample was added with 2 mL ethanol/pyrogallol (1%) and heated in a water bath at 70 °C for 2 min. Saponification was performed with 1 mL 12 N KOH for 30 min at 70 °C. Samples were extracted twice with n-hexane. Hexane phase was evaporated to dryness, suspended in absolute ethanol and filtered through a 0.45 µm micropore nylon membrane before injection. Tocopherols were analyzed according to the modified method described by Rossetti et al. (2010) using HPLC with a Dionex Ultimate 3000 system (DIONEX Corp., Sunnyvale, CA, USA) equipped with an Uptispher C18-HDO column 250x4.6 mm, 5 µm (Interchim, Montluçon, France). The injection volume was 20 µl. The elution was performed isocratically with ethanol/methanol 60/40 (v/v) at a flow rate of 0.8 mL/min at 25 °C. Detection was performed with a fluorescence detector at 296 nm and 330 nm.

4.1.2.10 Fatty acid composition

Fatty acid composition was determined as described by Piombo et al. (2006). Oil samples (10 mg) were added with 3 mL sodium methylate solution with phenolphthalein in rounded bottom flasks. Hydrochloric methanol (3 mL) was added until phenolphthalein discoloration was observed. Hexane (8 mL) and water (10 mL) were added. The organic phase was recovered, dried over anhydrous sodium sulfate,

and filtered for subsequent GC analysis using an Agilent 6890 chromatograph (Agilent, Santa Clara, CA, USA) equipped with an Innowax capillary column 30 m x 0.32 mm x 0.25 µm (Agilent, Santa Clara, CA, USA). Fatty acid methyl esters (FAME) were directly injected into the GC (He 1 mL/min, split 1:80). Injector and FID detector temperatures were 250 °C and 275°C respectively. The oven was heated from 185 to 225 °C at 5°C/min and held at 225 °C for 20 min. Fatty acids were identified by comparing their retention times with the FAME standards (Sigma-Aldrich, St. Louis, Missouri, USA).

4.1.2.11 Amino acid composition

Free amino acids were analyzed following the method used by Moore et al., (1958) Total amino acid analysis was performed using a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK). This system uses ion exchange chromatography with post-column ninhydrin derivatization and photometric detection with dual-wavelength measurements. The amino acid separation along the cationic column was obtained with a succession of four sodium citrate buffers of increasing pH (2.6 – 8.6) and ionic strength (0.2 – 0.5 M) and with an increasing temperature gradient (52 – 95 °C). Amino acids were derivatized with the ninhydrin reagent (135 °C) and detected simultaneously at 570 nm and 440 nm. The entire process lasted 90 min per sample, including the resin regeneration phase. Quantification was performed by comparing peaks areas with a complete standard including 26 amino acids acidic, neutral and basic amino acids (Sigma, St. Louis, Missouri, USA). Norleucine (250 nmol mL^{-1} in sodium citrate buffer, 0.2 M, pH 2.2) was also used as an internal standard.

4.1.2.12 Proteomic profiling

Jicaro seeds (10 g) were ground under liquid N_2 using a mortar and pestle. Frozen pulverized plant material (300 mg) was divided in two aliquots and extracted with 500 µL 0.1 M Tris-HCl buffer (pH 6.8). After centrifugation (10,000×*g* for 5 min, 4 °C), the supernatants of both aliquots were combined and used for protein fractionation. Total protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Berkeley, CA, USA) with bovine serum albumin (BSA) standards.

The crude extract (1 mg in 200 µL) was fractionated using reverse-phase high-performance liquid chromatography on a C_{18} column 4.6 x 250 mm, 5 µm particle diameter (Teknokroma Barcelona, Spain) with an Agilent 1200 chromatograph

monitored at 215 nm (Agilent, Santa Clara, CA, USA). The flow rate was 1 mL/min, and the elution was performed by applying a gradient from 0.1% trifluoroacetic acid (TFA) in water (solution A) to acetonitrile containing 0.1% TFA (solution B) as follows: 0% B for 5 min, 0–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min, as previously described by Lomonte et al.,(2014). The fractions were collected manually and evaporated by vacuum centrifugation (Eppendorf Vacufuge, Germany) at 45 °C. Once dried, the samples were reconstituted in water and mixed with reducing buffer for further separation by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% pre-cast gradient gels (Bio-Rad, Berkeley, CA, USA). Protein bands were stained with Coomassie G-250 and digitally recorded in a Chemidoc® imaging device (Bio-Rad). Then, protein bands were excised from the gels, reduced with dithiothreitol, alkylated with iodoacetamide, and digested with sequencing-grade bovine trypsin for 16 h in an automated digester (Progest, Digilab). Resulting peptides were mixed with an equal volume of a saturated solution of α-cyano-hydroxycinnamic acid in 50% acetonitrile with 0.1% TFA, and 1 μL of each peptide mixture was spotted onto an Opti-TOF 384-well plate, dried, and analyzed in positive-reflector mode using MALDI-TOF-TOF mass spectrometry model 4800 Proteomics Analyzer Plus instrument (Applied Biosystems, Foster City, CA, USA.). TOF spectra were acquired using 1500 shots and a laser intensity of 3000. The ten most intense precursor ions were automatically selected, and their TOF/TOF fragmentation spectra were acquired using 500 shots at a laser intensity of 3900. External calibration in each run was performed with CalMix® standards (ABSciex, Framingham, Massachusetts, USA) spotted onto the same plate. For protein identification, resulting spectra were searched against UniProt/SwissProt databases (general and Viridiplantae) using ProteinPilot® v.4 and Paragon® algorithm (ABSciex, Framingham, Massachusetts, USA) (≥ 95% confidence), and spectra were submitted to MASCOT public server (Matrix Science). Peptide sequence spectra with lower confidence scores were manually interpreted and searched using BLAST (http://blast.ncbi.nlm.nih.gov) for protein similarity and family assignment. A few tryptic digest samples were analyzed by direct infusion in nano-ESI-MS/MS using a QTrap3200 instrument (Applied Biosystems, Foster City, CA, USA) operated in positive mode. Doubly or triply charged peptide ions were fragmented by collision-induced dissociation, and the resulting MS/MS spectra were interpreted by manual *de novo* sequencing.

4.1.2.13 Trypsin inhibitor

Trypsin inhibitor activity was assessed according to the method described by Stauffer (1990). The determination was based on the decrease in the hydrolysis rate of substrates (added bovine trypsin) caused by the inhibitor. The method involves extraction at pH 9 of the inhibitors which were added with bovine trypsin. The activity of the remaining trypsin is measured with N-α-benzoyl-D,L-arginine p-nitroanilide hydrochloride (BAPNA) under standard conditions. The amount of p-nitroaniline formed during a 10 min incubation is measured spectrophotometrically, and absorbance values in its presence and absence are used to calculate the number of trypsin inhibitor units (TIU) per milligram of sample.

4.1.3 Results and discussion

4.1.3.1 Seed structure

As depicted **Figure 4.1** in the structure of jicaro seed consists of a brown seed coat, two white cotyledons stuck together and surrounded by a thin translucent envelope (cuticle). The seeds were small, thin, brown coated, and had an average weight of 53.3 ± 0.04 mg. The cotyledons represented 68% of the total seed weight. The seed coat, which accounts for approximately 32% of the seed weight, provides an effective protective layer.

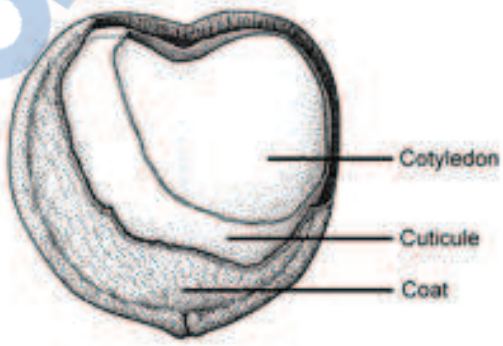

Figure 4.1. *Anatomy of jicaro seed (Crescencia alata)*

4.1.3.2 Main chemical composition

The main chemical composition of jicaro seed is shown in **Table 4.1.** The main characteristic of the whole seed was its high protein and lipid contents, comparable to values reported previously by Figueroa and Bressani (2000). Protein and lipid contents in jicaro cotyledon reached nearly 44 and 38% (d.w.) respectively. Similar protein

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contents were found in soybean cotyledon (**Table 4.1)** but lower lipid contents were reported by El Tinay et al., (1989). Therefore jicaro seed is defined as an oilseed by its high content of lipids and proteins as sunflower, rapeseed, cotton and peanut seeds (Rodrigues et al., 2012).

Table 4.1. Main chemical composition of jicaro seed

Chemical composition	Jicaro seed			Soybean
	Seed	Cotyledon	Coat	
Proteins (g/100 g)	33.4±0.4	43.7±1.2	15.8±0.7	42.0±0.3
Lipids (g/100 g)	32.8±0.11	38.1±0.2	4.18±0.38	19.8[d]±0.8
Sugars (g/100 g)				
Sucrose	5.15±0.03	5.5±0.03	2.04±0.78	0.94-3.2[a]
Fructose	0.42±0.02	0.50±0.05	0.11±0.06	0.06-0.2[a]
Galactose	0.06±0.00	-	0.04±0.02	0.007-0.02[a]
Glucose	0.03±0.00	0.01±0.01	0.04±0.01	0.068-0.11[a]
Stachyose	0.11±0.01	0.14±0.01	<0.10	3.43±0.12
Raffinose	<0.10	<0.10	<0.10	6.62±0.02
Verbascose	<0.10	<0.10	<0.10	0.13±0.01
Fiber (g/100 g)				
Neutral detergent fiber (NDF)	23.6±0.4	13.8±0.2	71.2±1.0	11.4±0.2
Cellulose	7.3±0.3	3.3±0.1	34.2±0.6	2.6±0.1
Hemicellulose	8.8±0.4	8.3±0.2	19.4±1.0	5.9±0.2
Lignin	7.5±0.03	2.2±0.01	17.6±0.01	2.9±0.01
Minerals (g/100 g)				
Phosphorus	0.93±0.01	1.24±0.01	0.48±0.02	0.61±0.01
Potassium	0.55±0.01	0.71±0.01	0.35±0.01	1.97±0.02
Magnesium	0.41±0.003	0.52±0.01	0.23±0.01	0.23±0.003
Calcium	0.06±0.002	0.05±0.02	0.11±0.03	0.18±0.004
Sulfur	0.35±0.01	0.45±0.01	0.21±0.01	0.04±0.003
Sodium	0.01±0.001	0.03±0.03	0.01±0.0003	0.01±0.002
Trace minerals (mg/kg)				
Iron	159.4±4.4	90.9±4.1	228.9±48.5	55.5±2.2
Zinc	72.7±2.2	117.4±42.1	43.8±1.3	46.0±0.9
Copper	31.6±4.3	43.5±6.0	22.2±0.2	16.4±0.7
Manganese	24.7±0.4	32.1±8.4	35.9±0.9	27.2±0.9
Others micro - constituents				
Pythic acid (mg IP6/100 g d.w.)	1325±72.5	1032±61	486±53	1760[c]±8.0
Polyphenols (mg GAE/100 g d.w.)	201±8.3	326±32.3	218±21.9	268[b]±47
Tannins (mg TAE/100 g d.w.)	0.16±0.01	0.15±0.01	0.15±0.01	93[b]±20
γ -Tocopherols (mg/kg d.b)	11.3±0.13	8.82±0.02	nd	15.92±0.23
δ/β-Tocopherols (mg/kg d.b)	1.06±0.14	1.15±0.72	nd	2.84±0.04
α-Tocopherols (mg/kg d.b)	nd	0.45±0.03	nd	5.45±0.06

Results are expressed in dry weight basis as means ± standard deviations of three replicates
nd: not determined.
[a](Song et al., 2013); [b](Malenčić et al., 2012); [c](Pedrosa et al., 2012); [d](El Tinay et al., 1989)

Sugar composition of jicaro seeds is shown in **Table 4.1**. Sucrose was the major sugar component followed by fructose (5.15 and 0.42 g/100 g d.w. respectively in the seed, and 5.5 and 0.50 g/100 g d.w. respectively in the cotyledon). Sugar composition in

soybean was determined in eight varieties using NMR proton nuclear magnetic resonance (^{1}H-NMR spectroscopy). The results show that sugar content depends on the variety and they showed low concentrations with respect to jicaro, except for glucose that is higher in the range 0.06 to 0.11% (Song et al., 2013). Another study on almond cultivars from Mallorca and California almond varieties also confirmed that sucrose is the major sugar component in kernels (Egea et al., 2009; Yada et al., 2013). Sucrose in peanuts accounted for the major fraction with a mean value of 4.6% (Bishi et al., 2013) Oligosaccharides (e.g. stachyose, raffinose and verbascose), which are considered indigestible (Wongputtisin et al., 2015), were not found in jicaro seed (less than 0.1 g/100 g d.w.). At the opposite, soybean contained high amounts of raffinose and stachyose (6.62 g and 3.43 g/100 g d.w. respectively), and also some verbascose (0.13 g/100 g d.w.).

Neutral detergent fiber (NDF) was the main constituent of seed coat with more than 70 g/100 g d.w. compared to only 13.3 g/100 g d.w. in cotyledon. Cellulose-like compounds were dominant in the seed coat and represented about half of fibers (34.2 g/100 g d.w.), followed by hemicellulose and lignin (19.4 and 17.6 g/100 g d.w. respectively). In the cotyledon, hemicellulose, cellulose and lignin accounted for 8.3 and 3.3 and 2.2 g/100 g d.w. respectively. These values are comparable to those found in soybean cotyledons (**Table 4.1**).

The total mineral content of jicaro cotyledon (3.3 g/100 g d.w.) was comparable to that of soybean (3.5 g/100 g d.w.) and similar to the contents reported for melon, pumpkin and gourd seeds (Adeyemi and Adediran, 1994). Jicaro cotyledon exhibited a significant concentration of phosphorus (1.24 g/100 g d.w.), potassium (0.71 g/100 g d.w.) and magnesium (0.52 g/100 g d.w.). Higher amounts of potassium were found in soybean cotyledon (1.97 g/100 g d.w.).

Phytic acid (PA) content was above 1000 mg/100 g of myoinositol hexakisphosphate (IP6). PA is a natural plant antioxidant constituting 1 to 5% of most cereals, nuts and oilseeds (Graf, 1990) and has the ability to chelate mono and bivalent metal ions, especially zinc, calcium, and iron. PA is the principal storage form of phosphorus in plant seeds. When iron and zinc bind to PA, they form insoluble precipitates and are far less absorbable in intestines. This process may therefore contribute to iron and zinc deficiencies in people whose diets rely on these foods for their mineral intake (Ruel and Bouis, 1998); (Hunt, 2003). However, health

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functionality of PA is still controversial, since some studies indicated a decrease of osteoporosis risk (López-González et al., 2013) and colon cancer with PA consumption via whole grains (Slavin, 2003).

Table 4.1 listed the main antioxidant compounds found in jicaro seed. Phenolic and tannin contents were low in all parts of jicaro seed. Higher values were found in soybean seed (Malenčić et al., 2012; Pedrosa et al., 2012). Tocopherols (γ-tocopherols δ/β-tocopherols and α-tocopherols) were quantified. γ-tocopherols is the most predominant form in the jicaro seed and cotyledons (11.3 to 8.82 mg/kg of dry weight, respectively) but relatively low concentrations if compared to soybean (15.92 mg/kg). Among tocopherols, α-Tocopherols is present in a low concentration (0.45 to 5.45 mg/kg) in jicaro cotyledons and soybean respectively, because the tocopherols' composition and other antioxidant compounds (polyphenol and tannins) their contents depend most often on the species, the genetic variability and the effect of environmental stresses on the growth and development of the seed (Britz and Kremer, 2002; Kumar et al., 2009; Tuberoso et al., 2007).

4.1.3.3 Fatty acid composition

Fatty acid profile of jicaro seed is shown in **Table 4.2**. Monounsaturated oleic acid (56.5% of lipid fraction) was the predominant fatty acid in jicaro cotyledon. Similarly high contents were observed in almond oil (62–80%) (Yada et al. 2011). The second most abundant fatty acid was polyunsaturated linoleic acid at almost 20% of the lipid fraction. In soybean, the reverse was observed with 22.2 and 52.5% of oleic and linoleic acids respectively. Saturated fatty acids such as palmitic acid and stearic acid represented 15 and 5% of jicaro lipid fraction, respectively. The content of polyunsaturated α-linoleic acid was only 2.5% in the jicaro lipid fraction. The other fatty acids were only present in trace amounts. Fatty acid profile of jicaro seeds from Nicaragua was similar to that obtained with jicaro seeds from Guatemala (Espitia-Baena et al., 2011). Jicaro seed was found to be rich in monounsaturated and polyunsaturated fatty acids, which accounted for more than 75% of the lipid content. Therefore, jicaro seed oil is potentially of high nutritional quality.

Table 4.2. Fatty acid composition of jicaro seed

Fatty acid	Jicaro			Soybean[a]
	Seed	Cotyledon	Coat	Cotyledon
Palmitic acid (16:0)	14.9±0.55	15.3±0.57	15.1±0.24	10.3±0.02
Stearic acid (18:0)	5.4±0.36	5.2±0.09	6.07±0.22	4.3±0.04
Arachidic acid (20:0)	0.74±0.13	0.70±0.03	0.82±0.07	-
Behenic acid (22:0)	0.33±0.10	0.29±0.03	0.38±0.02	-
Lignoceric acid (24:0)	0.24±0.08	0.22±0.08	0.30±0.04	-
Saturated fatty acids	**21.61**	**21.71**	**22.67**	
Palmitoleic acid (16:1, n-7)	0.45±0.02	0.50±0.02	0.57±0.04	-
Oleic acid (18:1, n-6)	55.3±0.93	56.5±0.70	54.1±0.57	22.2±0.61
Linoleic acid (18:2, n-6)	19.5±0.88	18.3±0.08	19.7±0.53	52.5±0.36
α-Linolenic acid (18:3; n-3)	2.4±0.23	2.3±0.03	2.4±0.12	7.3±0.12
Unsaturated fatty acids	**77.65**	**77.60**	**76.77**	

Results are expressed in g/100g oil as means ± standard deviations of three replicates.
[a](Slavin, 2003).

4.1.3.4 Amino acid and protein compositions

The major amino acid found in jicaro cotyledons (**Table 4.3**) was the essential amino acid leucine with a concentration up to 2.58 g/100 g d.w (7.4% of total amino acids). This value is similar in soybean cotyledon (3.23 g/100 g d.w.). The percent composition of the other essential amino acids in jicaro cotyledons, such as phenylalanine, valine, histidine, threonine, and methionine, were similar to those obtained with soybean cotyledon. Only lysine appeared at a much higher level in soybean cotyledon than in jicaro. The composition of non-essential amino acids was also very similar to that of soybean although essential to non-essential amino acid ratio was slightly lower (0.44%) in jicaro cotyledon.

Table 4.3. Amino acid composition of jicaro seed

Amino acid	Jicaro			Soybean
	Seed	**Cotyledon**	**Coat**	**Cotyledon**
Essential amino acids (EAA)				
Leucine	7.2±0.45	7.4±0.10	7.4±0.2	8.0±0.07
Phenylalanine	5.2±0.30	5.4±0.2	4.6±0.10	5.4±0.007
Valine	3.9±0.95	4.2±0.3	5.3±0.50	4.9±0.04
Histidine	3.3±0.66	2.9±0.1	3.1±0.01	2.8±0.0004
Threonine	3.1±0.15	3.1±0.1	3.2±0.1	3.7±0.07
Isoleucine	3.0±1.04	3.2±0.3	4.3±0.8	4.7±0.01
Lysine	2.4±0.12	2.5±0.1	3.0±0.7	6.3±0.21
Methionine	1.8±0.28	1.8±0.2	1.6±0.9	1.3±0.002
Total EAA	**29.9**	**30.6**	**32.6**	**37**
Non-essential amino acids (NEAA)				
Glutamic acid	22.2±1.51	22.0±0.5	20.4±0.4	19.2±0.09
Tyrosine	4.5±1.27	3.8±0.1	4.0±0.1	4.0±0.05
Arginine	14.8±0.35	16.3±0.3	12.7±0.4	8.1±0.26
Aspartic acid	8.0±0.66	7.7±0.01	7.9±0.3	11.8±0.14
Glycine	5.4±0.47	5.0±0.04	6.3±0.2	4.4±0.04
Alanine	4.7±0.39	4.6±0.01	4.9±0.1	4.5±0.05
Serine	4.4±0.77	4.2±0.3	4.1±0.2	4.7±0.12
Proline	4.2±0.40	3.9±0.2	4.5±0.1	5.3±0.01
Cysteine	2.0±0.56	1.9±0.4	2.6±0.04	0.9±0.07
NEAA	**70.1**	**69.4**	**67.4**	**63**
EAA/NEAA	**0.42**	**0.44**	**0.47**	**0.58**

Results are expressed in dry weight basis as means ± standard deviations of three replicates.

4.1.3.5 Proteomic analysis of jicaro cotyledon

Proteomic profiling was made for the first time on jicaro seeds. The crude protein extract of jicaro cotyledon which was extracted with no detergent and reducing agent, was resolved into 38 peaks via RP-HPLC (**Figure 4.2 (a)**). The peaks that eluted first (with a retention time < 22 min) did not correspond to proteins, as shown by SDS-PAGE analysis (**Figure 4.2 (b))**, and are probably metabolites or peptides, which are expected to elute early in the chromatogram. Protein peaks were further separated by SDS-PAGE under reducing conditions, resolving into more than 45 distinct protein bands that ranged from 10 to 110 kDa **Figure 4.2 (b))**. The higher diameter of the spots at lower molecular weights (MW) observed in SDS-PAGE gel indicates a major proportion of low MW proteins in jicaro cotyledon (~10 kDa). Soybean storage proteins which are composed of two major components, β-conglycinin (7S globulin) and

glycinin (11S globulin) have higher MWs (150 - 200 and 300 -380 kDa respectively). After extraction in the presence of a detergent and a reducing agent, SDS-PAGE showed that β-Conglycinin was composed of three subunits with MWs of 80 kDa, 75 kDa and 50 kDa respectively and glycinin was composed of six subunits, each made up of an acidic polypeptide chain with a MW of 34 - 40 kDa linked by a disulfide bond to a basic polypeptide chain with a MW of 15 - 20 kDa (Medic et al., 2014; Mujoo et al., 2003; Yaklich, 2001).

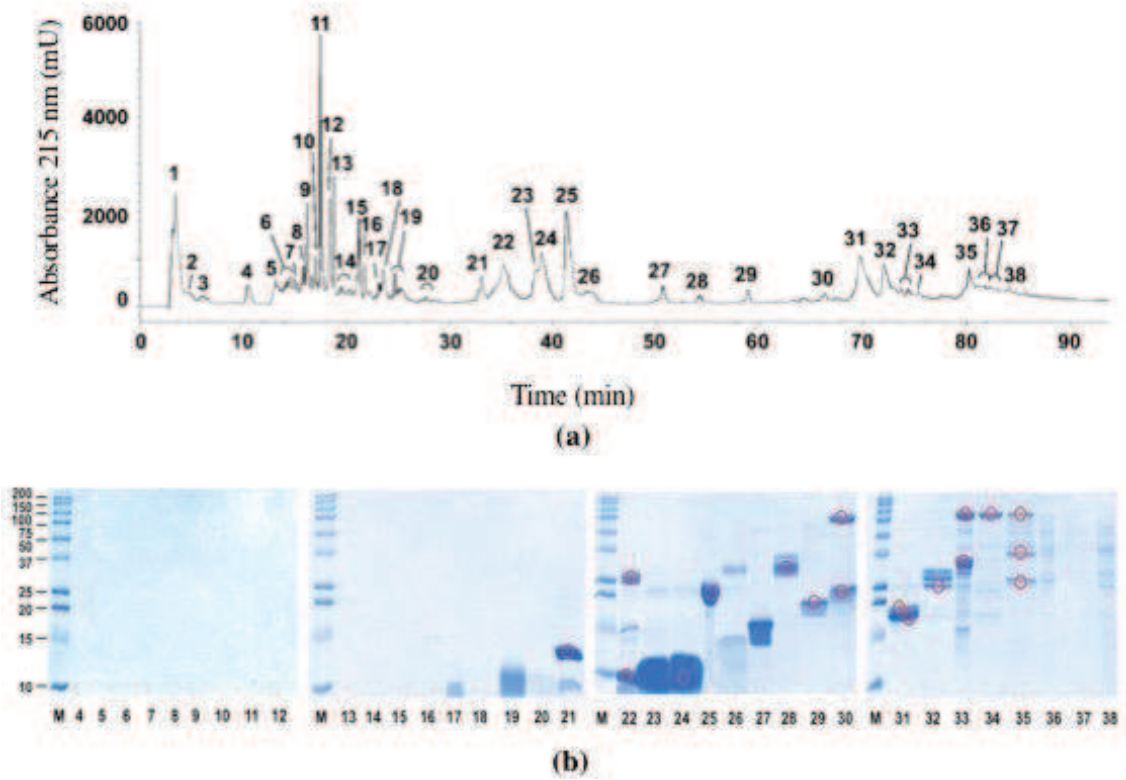

Figure 4.2. *Separation of jicaro seed proteins via RP-HPLC (a) followed by 15% SDS-PAGE of the resolved peaks (b). The bands with positive identification are labelled with red circles in b. "M" in figure b is the MW marker.*

A MALDI-TOF-TOF analysis of the trypsin-digested bands provided some peptide sequences that matched, with confidence levels of over 95%, those of known plant proteins reported in international databases (**Table 4.4**). The similarity of jicaro protein sequences to those from other plant genera facilitated their assignment to protein families associated with particular biological activities.

The sequence of the protein band that dominated in jicaro cotyledon and had an average MW of 11 kDa (peak 24, **Table 4.4**) matched the 2S albumin sequence. As storage proteins, the 2S albumins are water-soluble and highly abundant proteins used

by the plant as a source of nutrients, mainly nitrogen and sulfur, during germination (Agizzio et al., 2006).

As shown in **Table 4.4**, the other protein families identified by sequence similarity are specific to embryogenesis, but heat shock proteins were also detected, which may explain the exceptional resistance of jicaro to thermal and other environmental stresses such as drought (Coelho and Benedito, 2008); (Wang et al., 2004).

Proteomic analysis also revealed enzymes such as glucose and ribitol dehydrogenase homolog-1 and aldose reductase. These enzymes were found in cotyledon seeds and are involved in carbon metabolism and acquisition of desiccation tolerance, which are characteristics of seed development (Gallardo et al., 2003). It is worth noting that none of the sequences matched the trypsin or chymotrypsin-inhibitor sequences reported in the international database. This observation was consistent with jicaro seed antitrypsin activity which was found to be very low (< 0.1 TIU/mg), in contrast with soybean (43.5 TIU/mg). This low value of antitrypsin activity in jicaro seeds may enhance the digestibility of protein and the bioavailability of amino acids (Sarwar et al., 2012).

For the different protein sequences reported in **Table 4.4**, it was not possible to find matches with already reported sequences in international databases. Jicaro is a non-model organism for which there is no information currently available in these databases.

4.1.4 Conclusions

The characterization of jicaro seed (*Crescentia alata*) composition was completed, and these results highlighted the high potential of this novel, biodiverse food source due to its high oil and protein content. Another nutritional interest is its fatty acid and amino acid profiles but also the absence of anti-nutritional factors (anti-trypsin and α-galactosides). Nutritional characteristics of jicaro seeds were found very similar to those of soybean. In addition to soybeans, other oilseeds produce valuable products due to their high content of protein and oil, therefore jicaro might be a substitute for these imported seeds and promote the value of local resources in these regions of Central America. Jicaro trees which grow in nutrient-poor savanna soils and extreme climates, could provide a high-quality food source to local population. Food industries typically ignore local food resources for developing new products and prefer more globalized

food commodities, such as soybeans. Jicaro seeds might be used as raw material to develop innovative processed foods, such as a vegetable milk with very interesting sensory properties that would be of great interest to people allergic to lactose.

Acknowledgements

The authors wish to thank Gilles Morel, Joël Grabulos, Nathalie Barouh and Graziëlla Albaut from CIRAD for their excellent technical support. The present study was supported by PRESANCA II/CSUCA grant no. C-15-2012 and the French Cooperation in Central America (Institut Français pour l'Amérique Centrale-IFAC).

References

References of the article have been removed as they are accessible at the end of the thesis.

Table 4.4. Protein matches obtained from peptide sequences of fractionated jicaro seeds via MALDI-TOF-TOF mass spectrometry

HPLC peak	Mol wt (kDa)	Peptide ion		Amino acid sequence	Protein match; Species	Conf (%)	Sc	Related protein accession code[a]
		m/z	z					
21	14	1623.9	1	E[ca]EEGVEIDESKFR	Embryonic-like protein GEA6; *Arabidopsis thaliana*	99.0	9	~ Q02973
		1497.7	1	EQLGTEGYQQMGR		99.0	16	
		1339.7	1	SLEAQQ[da]HLAEGR	EMB-1 protein; *Daucus carota*	99.0	20	~ P17639
22a	26	1192.6	1	QTDEYGNPIR	Dehydrin DHN1; *Avicennia marina*	99.0	11	~ A8CVF3
24	11	2032.1	1	RALAALIRP[ox]SSHQQRRR	Uncharacterized protein; *Glycine max*	97.6	7	~ XP_003546585
		768.4	2	RGEEQCQCEALR	**2S albumin seed storage protein**; *Juglans nigra*	man	man	~ AAM54365
25	23	1167.7	1	RTRQPPEKR	Predicted protein; *Physcomitrella patens*	man	man	~ XP_001755540
28	32	1732.9	1	DGNGFITAAELAHSMAK	Ca^{2+}-binding protein CML17; *Arabidopsis thaliana*	95.6	9	~ Q9LQN4
29	19	1613.9	1	VDWRETPEAHVFK	17.4-kDa class I heat shock protein; *Arabidopsis thaliana*	99.0	9	~ P19036
		2143.2	1	EEVKVEVEDGNILQISGER		99.0	17	
		2271.3	1	KEEVKVEVEDGNILQISGER		99.0	18	
		2951.7	1	ADVPGLKKEEVKVEVEDGNILQISGER		99.0	14	
		2512.5	1	ASMENGVLTVTVPKEEVKKPEVK	17.8-kDa class I heat shock protein; *Solanum lycopersicum*	99.0	20	~ AAD30453
30a	96	1243.6	1	AGEYKDYAAQK	Embryonic protein DC-8; *Daucus carota*	99	16	~ P20075
		1274.6	1	TGEYKDYAAEK				
30b	20	1801.9	1	QEEDDFFTSEGN[da]VQR	G patch domain-containing protein 8 isoform X3; *Musa acuminata*	99.0	9	~ M0T1Q6
		999.6	1	AGVLENIKR	Hypothetical protein 231423; *Selaginella moellendorffii*	96.4	7	~ XP_002970024
31a	18	1808.1	1	ETPEAHVFK[fo]ADLPGLR	17.6-kDa class I heat shock protein; *Arabidopsis thaliana*	99.0	15	~ P13853
31b	17	1931.1	1	ASMENGVLTVTVPKEEVK	17.5-kDa class I heat shock protein; *Glycine max*	99.0	7	~ P04794
		974.6	1	FRLPENAK		74.1	9	
		1110.6	1	SLIPSFFSGR	18.2-kDa class I heat shock protein; *Vitis vinifera*	99.0	12	~ XP_002281285
		1794.1	1	ETPEAHIFKADLPGLR	Predicted protein; *Physcomitrella patens*	99.0	14	~ A9SJI0
		1641.9	1	IDWRETPEAHIFK		99.0	8	
32c	30	1211.7	1	LSFLYPGTTGR	Uncharacterized protein; *Physcomitrella patens*	99.0	12	~ A9RJ06
		1452.9	1	LKLSFLYPGTTGR		99.0	14	
		1105.7	1	I[pa]DTNGVIMR	Transcription repressor protein-related; *Musa balbisiana*	99.0	11	~ ABF70137
		2121.1	1	PGLTLGDVVPDLELDTTHGK	1-Cys peroxiredoxin B; *Oryza sativa*	99.0	11	~ P0C5D0
33a	110	1788.0	1	GAEMTNKEQNAKNHSK	Uncharacterized protein; *Selaginella moellendorffii*	99.0	10	~ D8TFU6
		1341.7	1	AALLLQ[da]ELGLSGR	Hypothetical protein I004822; *Eucalyptus grandis*	95.6	11	~ KCW54529

		1358.8	1	ELRATGELENAR	Cellulase-like protein; *Arabidopsis thaliana*	Masc	28	~ CAB83158
		1230.7	1	MASNLLKALIR	NADH dehydrogenase (ubiquinone) Fe-S protein; *Arabidopsis thaliana*	Masc	27	~ NP_566191
		1492.8	1	ETEMLESLTRKR	Uncharacterized protein; *Musa acuminata*	96.4	9	~ M0TCX2
33b	35	2041.1	1	AVGYCFASEGATVAFTYVK	Glucose and ribitol dehydrogenase homolog 1; *Arabidopsis thaliana*	99.0	12	~ Q9FZ42
		1263.7	1	P^{ca}SATQVNNYGR	Predicted protein; *Populus trichocarpa*	95.2	10	~ XP_002304281
34	110	1358.8	1	CPQLEELSLNR	Uncharacterized protein; *Physcomitrella patens*	99	13	~ A9TQ22
		1326.8	1	AREAASRELPVK	Predicted protein; *Physcomitrella patens*	97.2	9	~ A9S3R4
		1239.5	1	FGSVQSPSSSTR	Thioredoxin Y2 protein; *Arabidopsis thaliana*	Masc	30	~ NP_175021
35a	110	1680.9	1	NAVVTVPAYFNDSQR	Heat shock 70-kDa protein; *Zea mays*	99.0	9	~ P11143
35b	40	1260.7	1	IWCTNLAPER	Aldose reductase; *Hordeum vulgare*	99.0	12	~ P23901
35c	27	1927.1	1	HFVAVHGVGHGAWVYYK	Alpha/beta hydrolase fold superfamily; *Gentiana triflora*	99.0	25	~ A5A7N5

** Cysteine residues determined in MS/MS analysis are carbamidomethylated. MW: SDS-PAGE apparent mass, in kDa. Confidence (Conf, %) and score (Sc) values were calculated by ProteinPilot® v. 4.0 using Paragon® algorithm. Matches identified by MASCOT public search engine are indicated with probability values provided by the server. Possible but unconfirmed or ambiguous amino acid modifications suggested by the automated identification software are shown between brackets with the following abbreviations: ^{ox}: oxidized; ^{da}: deamidated; ^{fo}: formylated; ^{pa}: propionamide; ^{ca}: carbamidomethylated. Man: de novo amino acid sequencing by manual spectrum interpretation.*

[a] according to InterPro protein sequence analysis and classification (EMBL-EBI) at www.ebi.ac.uk.

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4.2 Key odor and physico-chemical characteristics of raw and roasted jicaro seeds (*Crescentia alata* K.H.B.)

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Abstract

Jicaro seeds (*Crescentia alata*) are widely consumed in Central America, primarily as a popular tasty and nutritious beverage called "horchata". Seeds are roasted to develop a specific aroma through a process that has never been explored. Volatile compounds, extracted from raw and roasted jicaro seeds (140 °C for 140 s) by SAFE (Solvent Assisted Flavor Evaporation), were analyzed by Gas Chromatography/Mass Spectrometry (GC/MS). Twenty-seven volatile compounds were isolated, among which, ethyl-2-methylbutyrate was designated by olfactometry as providing the characteristic jicaro note (0.16 and 0.47 mg/kg dry basis (d.b.) in raw and roasted seeds, respectively). The release of volatile compounds from the Maillard reaction, such as pyrazines, and the increase of ethyl-2-methylbutyrate after roasting, exhausted the pleasant jicaro aroma.

This mild roasting process had a slight impact on polyphenol, fructose and free amino acid contents, in agreement with the Maillard reaction. Confocal microscopy showed the coalescence of lipids in roasted jicaro seeds, which might explain the higher extracted fat content.

Keywords

Jicaro seeds, roasting, volatile compounds, SAFE, olfactometry, physico-chemical properties

4.2.1 Introduction

Jicaro (*Crescentia alata*), a species of the Bignoniaceae family, is a small tree native to Mexico and Central America and is widespread in dry regions of Central and South America (Bass, 2004) as well as India (Madhukar et al., 2013). The jicaro fruit has a lignified pericarp with many seeds embedded in a sweet pulp. The jicaro seed structure consists of a white cotyledon surrounded by a dark brown coat. It has been reported that jicaro cotyledon is rich in protein (43 g /100 g dry basis) and fat (38 g /100 dry basis) content. In addition, the seeds do not contain anti-nutritional factors (Corrales et al., 2017a).

Jicaro seeds are used in the preparation of one of the most consumed traditional beverages in Central America (mainly Nicaragua, Honduras and Salvador). This beverage presents pleasant sensory properties with high caloric value and has been part of school nutrition programs in Central America (Gutiérrez, 2012). During homemade preparation of the beverage, the seeds are roasted in mild conditions (90-110 °C during less than 5 min) (personal information) to develop a specific flavor, which is a key step. It is well known that roasting induces changes in chemical and physical properties that are more or less intense depending on both roasting time and temperature. High temperature and long roasting times decrease the content of unsaturated fatty acids (Yen, 1990) and the solubility of proteins (Davis et al., 2010). In addition, roasting induces changes in the internal microstructure and texture of the roasted product (Young and Schadel, 1993). Additionally, the characteristic and pleasant flavor of roasted products results from the formation of numerous volatile compounds (aldehydes, ketones, alcohols and pyrazines) (Xiao et al., 2014). Qualitative and quantitative determination of volatile compounds in foods requires high resolving power methods (Esteban et al., 1996). Solvent assisted flavor evaporation (SAFE) (Engel et al., 1999) and headspace solid-phase micro extraction (HS-SPME) (Pawliszyn, 1997) are among the common methods adopted for the analysis of volatile compounds. Another effective tool for the discrimination of odor components from a complex mixture is the combination of olfactometry and gas chromatography (GC-O) (D'Acampora Zellner et al., 2008). Odor compounds were studied using SAFE along with SPME-GC–O for the discrimination of key odorants.

In the case of jicaro seeds, traditional roasting is relatively mild and atypical, both in temperature and time compared to other seeds which can exceed 200 °C and take

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more than 40-45 minutes. Coffee roasting is usually conducted at higher temperature and longer time (180-250 °C during 12-60 min for light-dark roast) (Franca et al., 2005; Moon and Shibamoto, 2009) to get the particular roasted color and flavor required (Amaral et al., 2006; Kahyaoglu and Kaya, 2006). Although the composition of the raw seed has been published recently (Corrales et al., 2017a), no information exists about the impact of mild roasting on the seed. Additionally, neither volatile compounds naturally present in jicaro seed before and after roasting have been explored. The aim of this work was to characterize the profile of the volatile compounds of raw and roasted jicaro seeds obtained in mild conditions (140 °C for 140 s). The effect of this roasting on some physico-chemical characteristics of the seeds and on the evolution of their aroma will be studied. In addition, the existence of Maillard reactions in our mild conditions was considered.

4.2.2 Materials and methods

4.2.2.1 Raw materials

Jicaro seeds (*Crescentia alata*, H.B.K.) were collected in the Pacific region of Nicaragua during the harvesting season in November 2013. The seeds were obtained traditionally as follows: breakage of the calabash, fermentation of the pulp, separation of seeds from the pulp, washing and sun drying. A total of 100 kg of dried seeds were collected from the northwestern region of Nicaragua (latitude: 12.66°N, longitude: 86.44°W, altitude: 115.7 m). Upon reception at the University of Leon, Nicaragua, the seeds were sieved to remove foreign material, washed again, and sun dried to obtain a unique homogenized lot, representative of the overall product.

4.2.2.2 Roasting process

Jicaro whole seeds (30 g) were conditioned at 15% of moisture content for 24 hours previous to the roasting. Roasting was performed in three batches of 30g, in a laboratory roaster (Probat - BRZ 2, Emmerich, Germany) with a strict temperature control at 140 °C for 140 s with continuous stirring.

4.2.2.3 Jicaro samples for analysis

The lot of jicaro whole seeds was named “whole raw seeds” in our paper. Part of it was manually dehulled and named “dehulled raw seeds”. Another part of it was directly roasted and named “whole roasted seeds”. A fourth part which was firstly roasted and then manually dehulled and named “dehulled roasted seeds”. These four

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LE NUMERO 1 MONDIAL DU MÉMOIRES

lots were frozen with liquid nitrogen and stored at -80 °C in glass bottles until analysis. Analyses were carried out in triplicate on samples from each of these four lots

4.2.2.4 Bulk density

The bulk density was calculated by the ratio of the seed mass and the volume occupied. Briefly, 50 g of seeds were weighed and transferred to a 500 mL graduated cylinder. Then, 200 mL of sand was added to the seeds. The cylinder was tapped carefully to settle the sand.

4.2.2.5 Chemical analyses

Raw and dehulled roasted seeds were analyzed. Protein, lipid, polyphenol, soluble sugar, free amino acid and fatty acid contents were determined as previously described by Corrales et al., (2017a).

4.2.2.6 Confocal laser scanning microscopy

Raw and roasted cotyledons were examined using confocal laser scanning microscopy (CLSM) (inverted microscope NIKON Eclipse-TE2000-C1si NIKON, Champigny sur Marne, France), after dividing them longitudinally with a scalpel. The protein network and neutral lipids were stained, respectively, with a mix of Syto-9™ fluorescent dye (Kit LIVE/DEAD BacLight ™ Molecular Probes, Invitrogen) prepared in water to a concentration of 24 µM, and Nile red fluorescent dye (5H-benzo-R-phenoxazine-5-one, 9-diethylamino-; Sigma-Aldrich, St Louis, MO) prepared in 1,3-propanediol at a concentration of 0.1% w/v. These two solutions of fluorescent dyes were mixed immediately before observation at a 2:1 ratio of Syto 9™/Nile red. Then, 10 µL of this mixture were dropped onto the cotyledon slice and stored for 15 min at room temperature to allow time for diffusion. The dyed slices were observed with an oil immersion objective x 100 magnification. Confocal observations were performed using an argon laser operating at 488 nm excitation wavelength (emission was detected between 500 and 530 nm) and a He–Ne laser operating at 543 nm wavelength excitation (emission was detected between 565 and 615 nm) (Sadat-Mekmene et al., 2013).

4.2.2.7 Extraction of volatile compounds

Solvent-assisted flavor evaporation (SAFE)

Raw, roasted, and dehulled roasted seeds were ground with liquid nitrogen in a grinder (Moulinex AR1105, France). Then, 80 g of each sample was placed in a 500 mL Erlenmeyer spiked with 24 µg internal standard of trans-2-octenal (Sigma-Aldrich,

France). Samples were extracted twice with 150 mL of a pentane–ether (HPLC grade ≥ 99 %) mixture (1:1 v/v) (Sigma-Aldrich, France) under constant stirring and a delicate flow of gaseous nitrogen for 30 min prior to filtration over anhydrous sodium sulfate. The volatile fraction of the filtrate was isolated by high-vacuum distillation (4.5 10^{-3} mbar, during 120 min) using SAFE at 45 °C. Distillate with volatile compounds was trapped in a 500 mL flask cooled with liquid nitrogen. The solution was thawed and concentrated to approximately 500 µL in a Kuderna Danish glassware (KD) in a water bath at 45 °C. Blank experiments were performed previously to sample extractions to prevent errors in attribution of peaks provided by solvents. Extractions and analyses were conducted in triplicate.

Headspace Solid Phase Micro Extraction (HS-SPME)

Briefly, 1.5 g of roasted seeds were placed and sealed in 10 mL headspace vials and extracted by HS-SPME (Lebrun et al., 2008). Extraction was carried out at 60 °C with 15 min incubation, followed by 30 min trapping and shaking, using a polydimethylsiloxane/divinylbenzene fiber (PDMS/DVB 65 µm, SUPELCO, Bellefonte, PA, USA). Although focused on the odor perception, the analysis realized over a short time period, showed a good repeatability, over the 19 volatiles compounds kept, with coefficients of variation, calculated on peak area, moving from 4.42% to 8.89% with a mean of 6.91%.

4.2.2.8 Analyses of volatile compounds

Gas Chromatography/ Mass Spectrometry (GC/MS) of SAFE extracts

A tandem gas chromatograph 6890/MSD 5973N (Agilent Technologies, Palo Alto, USA) and a Gerstel autosampler MPS-2 were used. A non-polar capillary column DB-5MS (5% diphenyl, 95% dimethyl siloxane, 30 m x 0.250 mm x 0.25 µm) and a polar capillary column DB-Wax UI (polyethylene glycol 30 m x 0.25 mm x 0.25 µm), both from J&W Scientific (Folsom, CA, USA), were used with a carrier gas hydrogen flow rate of 1.2 mL/min. Then, 1 µL on-column injection at 43 °C was eluted with the following temperature program: 3 °C per min from 40 °C to 170 °C, then 10 °C per min up to 240 °C and held for 10 min. Mass spectrum were recorded in EI^+ mode at 70 eV within a range of 40 to 350 Da with solvent delay: 2 min and scan speed: 4.52/s. Analyzer and source temperatures were 150 °C and 250 °C, respectively. Data were analyzed with Masshunter version B. 06.00 (Agilent Technologies, Palo Alto, USA).

The identification of peaks was performed by comparing their mass spectra with those from the NIST 2011 (National Institute of Standard Technology) database. Co-injection on both columns of alkanes from C_8 to C_{20} (Sigma-Aldrich, St. Louis, MO, USA) was used to calculate Kovats retention index (RI) for comparison with those found in Flavornet, Pherobase and NIST websites.

Olfactory Detection Port (ODP) of SPME extracts

GC/MS analyses of SPME extracts were carried out with the conditions described above, using the same DB-Wax column and an Olfactory Detector Port 3 (ODP3, GERSTEL GmbH & Co. KG). Injection conditions used were splitless mode for 30 s at 250 °C and a split ODP/MSD flow ratio 1/1 with the ODP port at 200 °C. Data were recorded with Gerstel ODP recorder version 2.13.

A panel of ten untrained panelists (five women and five men of various ages, ranging from 28 to 55 years old) was recruited among the staff of the research unit at CIRAD, Montpellier, France. Each of panelists have been educated previously, to become familiar with the specific jicaro odor, by smelling ground roasted jicaro seed. Two sniffing sessions of 35 min each, after which no odor was detected, were conducted on different days. The panelists sat comfortably with their nose placed in the nosepiece of the ODP flushed with humidified air (100 mL/min) to avoid dryness of the nasal mucosa. While sniffing, the panelists were asked to give a qualitative perception and description of the fragrances of jicaro seeds using their own words. Comments were reported automatically on the chromatogram as peak annotations. The repeatability of experiments for olfactometric analyses was assessed after two sniffing sessions, for each panelist smelling volatile compounds (extraction duplicated), by measuring the percentage of similar responses for each volatile compound. The odor activity value (OAV), which is the ratio between the concentration of an individual compound and the perception threshold found in the literature, was calculated for ethyl-2-methyl butyrate.

4.2.2.9 Quantification and semi quantification of volatile compounds

Regarding SAFE extracts, the quantification of all volatile compounds was expressed as the trans-2-octenal equivalent, considering the ratio of response coefficients equal to 1. For the specific quantification of ethyl-2-methylbutyrate, calibration curves were carried out with a pure standard (Sigma-Aldrich, St. Louis, MO, USA). The determination of response coefficient was performed, based on extraction of

ion 57 as a quantifier and ions 102 and 85 as qualifiers. A three-point calibration curve was performed with a linear regression coefficient (R^2) of 0.9988.

4.2.2.10 Statistical analysis

The t-test was employed to ascertain the difference between raw and roasted jicaro seed for chemical composition. Significant differences were reported at $p < 0.05$.

4.2.3 Results and Discussion

4.2.3.1 Effect of roasting on physico-chemical properties of jicaro seeds

Mild roasting at 140 °C and 140 s caused changes in jicaro seeds. Jicaro seeds lost approximately 11.4% of their weight due to loss of water during roasting. Roasting resulted also in a quasi-total dehydration of jicaro seeds, with a moisture content decreasing from 15 to 2%. Simultaneously, seed density was reduced from 1.31 g/mL to 0.64 g/mL due to a gain in seed volume.

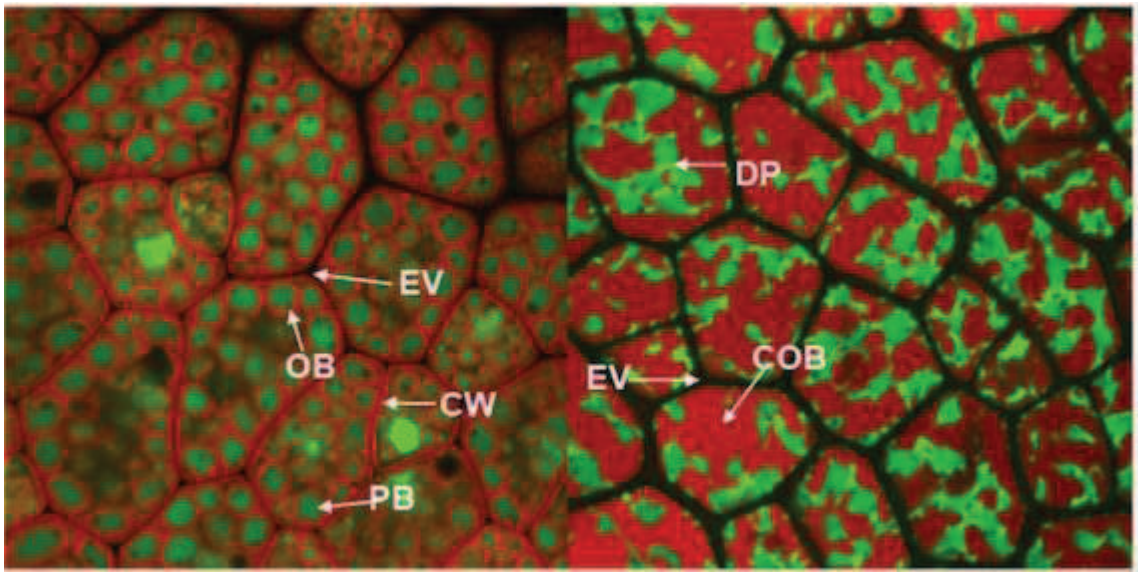

Figure 4.3. *Confocal images of dehulled jicaro seeds. OB= Oil Body; PB= Protein Body; COB= Coalescence Oil Body; DP= Denaturation of Proteins, CW= Cell Wall; EV= Extracellular Volume. (a) Raw and (b) roasted seeds showing coalescence of oil bodies and protein denaturation (DP) through mild roasting. Oil and protein bodies were stained in red and green, respectively.*

The prominent characteristic of dehulled raw seeds was their high protein and lipid contents, (Corrales et al., 2017a) which is comparable to those of other oilseeds (Purkrtova et al., 2008). Observation of raw jicaro seeds by confocal laser scanning microscopy showed a regular structure with spherical proteins bodies (PB) surrounded

by much smaller spherical oil bodies (OB) (**Figure 4.3 (a)**). After roasting, significant changes were observed, with more disaggregated cells and larger spaces between cells, possibly due to seed dehydration. A coalescence of oil bodies was observed, along with a likely disorganization of protein bodies due to their denaturation (**Figure 4.3 (b)**). Consequently, as a result of roasting, an improvement in lipid extractability (from 38.1% to 43.6% d.b) was observed (**Table 4.5**). This might be due to increased extraction efficiency of coalescent lipids during Soxhlet analysis Amaral et al., (2006) also reported that roasting produced a higher yield of oil extraction during standard methods of analysis with solvent. As a consequence, the bio-accessibility of lipids may also probably increased (Grundy et al., 2015).

The primary chemical compositions of dehulled raw and roasted jicaro seeds are shown in **Table 4.5**.

Table 4.5. Chemical composition of raw and roasted dehulled jicaro seeds

Chemical components (g/100 g d.b.)	**Dehulled raw seeds**	**Dehulled roasted seeds**
Proteins *	$43.7^{a} \pm 1.2$	$41.6^{b} \pm 0.19$
Lipids *	$38.1^{a} \pm 0.2$	$43.6^{b} \pm 1.80$
Polyphenols*	$0.33^{a} \pm 0.03$	$0.24^{b} \pm 0.02$
Sugars		
Sucrose	$5.5^{a} \pm 0.33$	$5.36^{a} \pm 0.13$
Fructose *	$0.50^{a} \pm 0.05$	$0.20^{b} \pm 0.03$
Glucose	$0.01^{a} \pm 0.01$	$0.02^{b} \pm 0.01$

Results are expressed as the means ± standard deviations of three replicates.
** Different superscript letters indicate significant differences ($p < 0.05$). Polyphenols expressed in Gallic Acid Equivalent (GAE)*

After mild roasting, as the proportion of extracted lipids increased, the content of proteins, some amino acids, fructose and polyphenols decreased (**Table 4.5**). The loss of free amino acids compared to their initial value ranged from 56% for cystine and 1% for proline (**Table 4.6**). Globally, free amino acids reduced from 2.36 to 1.72 mmol/100 g d.b. corresponding to a loss of 0.6 mmol. Similarly, if roasting had no significant influence on the content of sucrose and glucose (**Table 4.5**), the content of the most reducing sugar, fructose, decreased from 0.50 to 0.20% d.b, corresponding to a loss of 1.67 mmol/100 g. Both reductions of fructose and free amino acids were involved in Maillard reactions that occurred even during mild roasting conditions.

The total polyphenol content was also reduced during roasting from 0.33 to 0.24 g 100 g d.b. This could be either due to volatilization or degradation during thermal treatment.

Table 4.6. Free amino acid composition of dehulled jicaro seed before and after roasting

Free amino acids (mmol/100 g d.b.)	Dehulled raw seeds	Dehulled roasted seeds
Glutamic acid *	$0.56^{a} \pm 0.01$	$0.35^{b} \pm 0.01$
Arginine	0.46 ± 0.30	0.39 ± 0.30
Asparagine	0.24 ± 0.02	0.14 ± 0.01
Aspartic acid	0.18 ± 0.01	0.14 ± 0.02
Histidine	0.15 ± 0.01	0.09 ± 0.01
Tryptophan	0.09 ± 0.01	0.06 ± 0.01
Proline	0.09 ± 0.01	0.09 ± 0.01
Valine	0.09 ± 0.01	0.06 ± 0.01
Lysine	0.08 ± 0.01	0.09 ± 0.02
Alanine *	$0.07^{a} \pm 0.01$	$0.01^{b} \pm 0.01$
Glycine	0.06 ± 0.01	0.06 ± 0.01
Serine	0.06 ± 0.01	0.03 ± 0.01
Leucine	0.05 ± 0.01	0.04 ± 0.01
Phenylalanine	0.05 ± 0.01	0.04 ± 0.01
Threonine	0.05 ± 0.01	0.04 ± 0.01
Isoleucine	0.03 ± 0.01	0.03 ± 0.01
Cystine	0.03 ± 0.01	0.01 ± 0.01
Methionine	0.01 ± 0.005	0.01 ± 0.001

* *Different superscript letters indicate significant differences ($p < 0.05$).*

Dehulled raw seeds contained high amounts of unsaturated fatty acids (oleic and linoleic acids, and to a lesser extent α-linolenic acid, with 56.5, 18.3 and 2.3 g/100 g oil, respectively). The composition of saturated fatty acids did not change significantly after mild roasting. Even during tougher roasting conditions, no differences in saturated fatty acid composition were reported in safflower seeds (Lee, 2004). Regarding unsaturated fatty acids, only linoleic and alpha linoleic acids showed statistically significant changes, although in both cases it was a slight increase (**Table 4.7**). This could be explained by poorer extraction of unsaturated fatty acids in raw seeds. The same behavior was observed in *C. cathartica* seeds after thermal treatment, where linoleic and eicosadienoic acid appeared after roasting (Seena et al., 2005). Although our results were contrary to those of sesame seeds (Yen, 1990), the conditions were completely different: a mild roasting of jicaro seeds versus an extended roasting at high temperatures (> 260 °C during 30 min) in the case of sesame seeds.

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Table 4.7. Fatty acid composition (g/100 g oil) of dehulled jicaro seed before and after roasting

Fatty acids	Dehulled raw seeds	Dehulled roasted seeds
Saturated fatty acids	**21.71**	**21.74**
Palmitic acid (16:0)	15.3 ± 0.57	15.5 ± 0.17
Stearic acid (18:0)	5.2 ± 0.09	5.1 ± 0.11
Arachidic acid (20:0)	0.70 ± 0.03	0.65 ± 0.05
Behenic acid (22:0)	0.29 ± 0.03	0.28 ± 0.10
Lignoceric acid (24:0)	0.22 ± 0.08	0.19 ± 0.11
Unsaturated fatty acids	**77.60**	**77.37**
Palmitoleic acid (16:1, n-7)	0.50 ± 0.02	0.48 ± 0.03
Oleic acid (18:1, n-9)	56.5 ± 0.70	55.7 ± 0.24
Linoleic acid (18:2, n-6) *	18.3^{a} ± 0.08	19.1^{b} ± 0.27
α-Linolenic acid (18:3; n-3) *	2.3^{a} ± 0.03	2.6^{b} ± 0.07

Results are expressed as the means ± standard deviations of three replicates.
**Different superscript letters indicate significant differences ($p < 0.05$).*

4.2.3.2 Identification and quantification of volatile compounds

Chromatographic profiles of volatile compounds of jicaro seeds before and after roasting are presented in **Figure 4.4**. Twenty-seven volatile compounds were detected in raw and roasted jicaro seeds. SAFE quantification of the volatile compounds was reproducible with coefficients of variation between 0.3 and 13% and a mean of 5.3%. All volatile compounds found in jicaro seeds were reported previously in the literature for raw and roasted seeds (Ivanova-Petropulos et al., 2015; Ramli et al., 2005; Xiao et al., 2014) and were therefore tentatively identified by presenting good matching indices with NIST and RI databases on both polar and non-polar columns. Only ethyl-2-methylbutyrate was quantified using a commercial standard.

Most of the volatiles found in the roasted seeds were already present in raw seeds (19 out of 27) (**Table 4.8**). The other eight (8) compounds are typically generated during roasting: benzaldehyde, benzeneacetaldehyde, hexanal, 2,5-dimethyl pyrazine, trimethyl pyrazine, 3-ethyl-2,5-dimethyl-pyrazine, 2-ethyl-5-methyl pyrazine and dimethyl sulfone. Many of them were also found in roasted coffee (Nebesny et al., 2007).

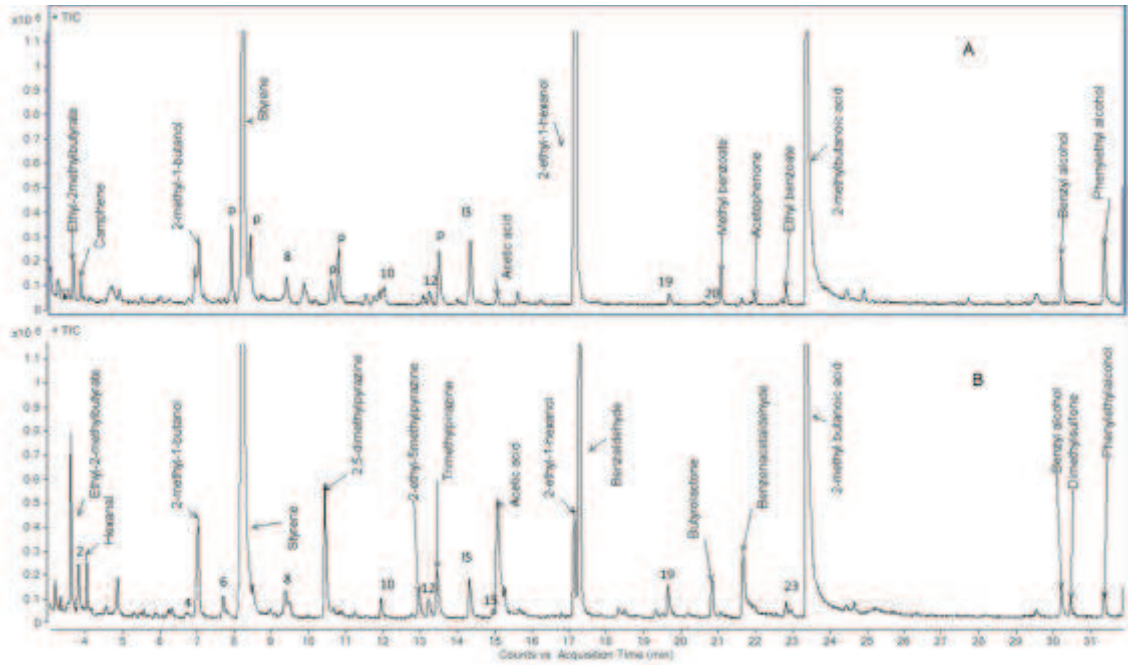

Figure 4.4. *Representative GC-chromatogram of jicaro seeds. A) Raw and B) Roasted at 140 °C x 140 s. Peak number referenced in table 4. Internal Standard (IS) and pollution (p)*

Most of the volatiles found in raw seeds increased their concentration after roasting, such as styrene, 2-methylbutanoic acid, acetic acid, 2-methyl-1-butanol, benzyl alcohol, ethyl-2-methylbutyrate, γ-butyrolactone, camphene, and 2-pentyl furan. All of them are derived from biosynthetic pathways of amino acids, fatty acids, phenolic and terpene compounds (Schwab et al., 2008; Yang et al., 2013). Terpenes are known to be naturally occurring as intense smell compounds in fatty products such as oilseeds (Bail et al., 2008). Methylbenzoate and ethylbenzoate were no longer detected after roasting, due to their high relative volatility and diffusivity in heating process (Fellows, 2000). After dehulling of roasted seeds, the contents of volatile compounds decreased or disappeared except pyrazines for which the levels increased.

Styrene, which contributes to the sweet and balsamic aroma note in jicaro seeds, was the most abundant volatile compound in jicaro seeds (16.25 mg/kg d.b.), and this compound increased significantly after roasting (29.44 mg/kg d.b.). A similar increase of styrene concentration was reported for cashews after roasting (Agila and Barringer, 2011). Styrene is common, as it is naturally synthesized by several plant species (Fernandez et al., 2005). Previous studies reported that styrene could be generated by decarboxylation of trans-cinammic acid (Sabatini et al., 2009).

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LE NUMERO 1 MONDIAL DU MÉMOIRES

In the case of pyrazines, trimethyl pyrazine was identified as having the highest concentration (1.49 mg/kg d.b.) in dehulled, roasted seeds. Importantly, besides the mild roasting conditions used, Maillard reactions induced the formation of pyrazines, which are often recognized for generating the typical roasted aroma (Cheong et al., 2013; Lykomitros et al., 2016). Free amino acids, proteins, fructose and polyphenols are the substrates in the thermal Maillard reaction that lead to the generation of pyrazines. Another consequence of roasting was a significant increase in the acetic acid concentration (0.95 mg/kg compared to 0.006 mg/kg in raw seeds). It is well known that acetic acid is formed by sugar degradation during Maillard reaction (Smuda and Glomb, 2013). Another identified compound, dimethyl sulfone, appeared only after roasting. This compound might be generated from a Strecker degradation product and derived from methionine (Murkovic et al., 2004).

Among the four identified aldehydes, benzaldehyde was the volatile compound with the highest concentration (3.53 mg/kg) in roasted seeds. Some studies showed that in sugar/phenylalanine model systems, benzaldehyde formed naturally at 175 and 200 °C during 20 s exposure to oxidative conditions (Fong and Yaylayan, 2008). Hexanal was also identified after roasting, as its generation was directly related to lipid oxidation of unsaturated fatty acids in roasted foods (Domínguez et al., 2014; Murkovic et al., 2004).

4.2.3.3 Determination of key aroma compounds by frequency of detection in olfactometry

It is well-known that during olfactory evaluation, the relative importance of volatile compounds in the formation of typical odors does not depend exclusively on concentration. Therefore, odor compounds responsible for the pleasant aroma of roasted jicaro seeds were assessed by SPME-olfactometry analysis and frequency of detection (**Table 4.8**). Twenty-two (22) aroma compounds were perceived in two independent tests by 10 untrained panelists, allowing creation of an odor profile for roasted jicaro seeds (**Table 4.8**). Ethyl-2-methylbutyrate was the only compound perceived by all of the panelists (100%) and was described as the typical aroma of jicaro seeds. In addition, ethyl-2-methylbutyrate showed a significant increase from 0.16 mg/kg in raw seeds to 0.47 mg/kg in roasted seeds. This ester has been identified previously as one of the most odor-active compound in pineapple and in criollo cocoa bean (Frauendorfer and

Schieberle, 2008; Tokitomo et al., 2014). In jicaro seeds, ethyl-2-methylbutyrate appeared to contribute most to the sweet smelling and apple-like odors.

The odor activity value (OAV) is linked to the relationship between the odor threshold and the concentration of volatile compounds, and it confirms the key contributions that volatile compounds have on the typical odor of roasted jicaro seeds. The OAV of ethyl-2-methylbutyrate was equal to 1808 (much greater than 1) taking an odor threshold reported in the literature of 0.26 ppb (Frauendorfer and Schieberle, 2008)

On the other hand, 95% of the panelists smelled the typical odor of pyrazines. Pyrazines are well known for being responsible for roasted aroma notes in roasted food (Siegmund and Murkovic, 2004). Additionally, dimethyl sulfoxide and dimethyl sulfide were perceived by 95% and 90% of panelists, respectively. These compounds were not detected in SAFE extract, likely because of the dilution factor and the solvent used. The presence of dimethyl sulfide has been reported in fermented foods such as tea (Zhu et al., 2015) and wine (Gürbüz et al., 2013). Volatile sulfur compounds might be derived from methionine following a series of biochemical transformations (Schwab et al., 2008). Despite the presence of sulfur in these compounds, there was no negative influence on the flavor of roasted jicaro seeds.

4.2.4 Conclusion

For the first time, volatile compounds were identified and quantified in raw, roasted and dehulled roasted jicaro seeds. Mild roasting was sufficient to develop typical aroma compounds of Maillard reaction, such as pyrazines, in jicaro seeds, which are thin and small. Most roasting processes require higher temperatures (> 200 °C) and longer durations (> 10 min) to develop the typical roasted flavor and color (Saklar et al., 2003). Likewise, microstructural changes were observed in jicaro seeds after this mild roasting. No noticeable losses of macronutrients were observed. GC-olfactometry analysis showed that ethyl-2-methylbutyrate was the key aroma compound in roasted jicaro seeds.

Acknowledgments

The authors wish to thank each panelist for their contribution to the olfactometry analysis. The present study was supported by PRESANCA II/CSUCA grant no. C-15-2012, the French Cooperation in Central America (Institut Français pour l'Amérique Centrale-IFAC) and the Europea Union Latin America Academic Links.

References

References of the article have been removed as they are accessible at the end of the thesis.

Table 4.8. Identification and semi-quantification (mg/kg d.b.)[b] on DB-wax of volatile compounds extracted by SAFE from whole raw seeds (Raw), whole roasted seeds (Roasted) and dehulled roasted seeds (Dehulled roasted), and odor compounds of whole roasted seeds detected by GC–O analysis

# Peak	Volatile compounds	RI[a] DB-wax		RI[a] DB-5		Raw	Roasted	Dehulled roasted	Detection frequency %	Odor threshold (ppb)	Odor descriptor
		Calc*	Lit*	Calc*	Lit*	SAFE	SAFE	SAFE	SPME/GC-O	SPME/GC-O	Literature; Panel
	Aromatic (1)										
7	Styrene	1221	1241[f]	884	893[f]	16.25±0.57	29.44±0.18	24.12±1.17	75	100 (Leonardos et al., 1974)	Balsamic [f]; Chemical
	Alcohols (5)										
16	2-ethyl-1-hexanol	1486	1487[f]	1030	1033[f]	6.60±0.19	0.73±0.00	0.20±0.01	65	246 (Schiffman et al., 2001)	Rose [f]; **Floral**
5	2-methyl-1-butanol	1185	1208[f]	700	711[n]	0.51±0.02	0.89±0.02	1.07±0.07			Wine [f]
26	Phenylethyl alcohol	1911	1908[v]	1074	1063[v]	0.31±0.01	0.19±0.01	0.18±0.03	55	17 (Schiffman et al., 2001)	Honey, floral [f]; **Candy**
25	Benzyl alcohol	1875	1865[f]	1040	1039[f]	0.18±0.01	0.25±0.00	0.25±0.02	55	20000 (Escudero et al., 2007)	Sweet, Floral [f]; **Medicament**
10	Hexanol	1331	1337[n]	863	867[p]	0.09±0.01	0.11±0.01	0.03±0.02	60	500 (Larsen and Poll, 1992)	Green [f]; **Herbal**
	Esters (4)										
20	Methyl benzoate	1605	1605[v]	1064	1070[n]	0.46±0.01	0.04±0.01	-			Herb, sweet [f]
8	2-methylbutyl-2-methylbutyrate	1256	1260[n]	1104	1103[v]	0.35±0.01	0.24±0.03	0.23±0.04			Apple [v]
1	Ethyl-2-methylbutyrate	1084	1069[p]	845	846[v]	0.16±0.01	0.47±0.03	0.24±0.01	100	0.26 (Frauendorfer and Schieberle, 2008); 0.006 (Zheng et al., 2012)	Apple [f]; **Jicaro**
23	Ethyl benzoate	1670	1673[n]	1130	1138[n]	0.16±0.01	0.12±0.01	-			Chamomile, flower [f]
	Organic Acids (3)										
24	2-methylbutanoic acid	1673	1667[p]	869	873[f]	1.47±0.02	6.04±0.02	1.68±0.17	90	180 (Larsen and Poll, 1992)	Cheese [f]; **Cheesy**
18	2-methylpropanoic acid	1560	1584[p]	755	758[n]	0.04±0.01	0.28±0.01	0.05±0.00			Cheesy, rancid [f]
15	Acetic acid	1426	1434[p]	598	600[v]	0.006±0.01	0.95±0.01	0.18±0.02	90	1000 (Leonardos et al., 1974)	Sour [f]; **Vinegar**
	Ketones (2)										
22	Acetophenone	1631	1645[f]	1041	1041[f]	0.75±0.01	-	-			Almond, floral [f]
19	γ-butyrolactone	1596	1602[n]	896	909[f]	0.25±0.02	0.41±0.04	-	85	35000 (Escudero et al., 2007)	Caramel [f]; **Flowery**
	Acetone	817	813 [n]			-	-	-	55	10000 (Leonardos et al., 1974)	**Flowery**
	Furan (1)										
6	2-pentyl furan	1206	1211[n]	983	993[f]	0.03±0.01	0.22±0.02	0.03±0.01			Butter [f]

	Terpenes (2)										
2	Camphene	1090	1075[f]	953	953[f]	0.13±0.01	0.34±0.04	0.24±0.01			Camphor [f]
4	D-Limonene	1176	1178[f]	1026	1033[f]	0.04±0.00	0.04±0.01	-			Lemon, orange [f]
	Aldehydes (4)										
17	Benzaldehyde	1491	1495[f]	955	960[f]	-	3.53±0.03	1.67±0.12			Almond [f]
21	Benzeneacetaldehyde	1622	1625[f]	1040	1049[f]	-	0.89±0.01	0.12±0.03			Honey, sweet [f]
3	Hexanal	1096	1084[f]	803	801[f]	-	0.32±0.03	0.11±0.002	55	4.5 (Larsen and Poll, 1992)	Grass [f]; **Herbal**
12	Nonanal	1370	1385[f]	1106	1104[f]	0.05±0.00	0.17±0.02	0.12±0.01			Soapy, fatty [f]
	Furfural	1444	1455[f]			-	-	-	70	776 (Schiffman et al., 2001)	Bread [f]; **Roasted**
	2-methyl butanal	907	910 [f]			-	-	-	60	12.5 (Suffet, 1995)	Malt [f]
	3-methyl butanal	910	912 [f]			-	-	-	50	0.15 (Suffet, 1995)	Cocoa, almond [f]
	Pyrazines (4)										
9	2,5-dimethyl pyrazine	1286	1290[n]	902	911[p]	-	1.01±0.05	1.32±0.08	95	1700 (Buttery, Orts, Takeoka, & Nam, 1999)	Roasted nut [f] Popocorn
13	Trimethyl pyrazine	1375	1395[v]	994	1002[v]	-	0.44±0.01	1.49±0.11	75	23 (Buttery et al., 1999)	Roasted [f]; **Roasted**
14	3-ethyl-2,5-dimethyl-pyrazine	1423	1435[f]	1080	1082[f]	-	0.30±0.03	0.38±0.03			Roasted, potato [f]
11	2-ethyl-5-methyl pyrazine	1361	1386[p]	992	1000[n]	-	0.24±0.01	0.37±0.02			Sweet [f]
	Sulfur (1)										
26	Dimethyl sulfone	1884	1833[p]	910	911[n]	-	0.17±0.02	0.20±0.01	50	-	Sulfur [f]
	Dimethyl sulfoxide	1567	1553[p]			-	-	-	95	-	Off-flavor
	Dimethyl sulfide	712	716 [f]			-	-	-	90	2.24 (Schiffman et al., 2001)	Sulfur [f]; **Sulfur**

[a] *RI: Retention index values*
[b] *Mean value ± SD*
[(f)] *www.flavornet.org,* [(p)] *www.pherobase.com,* [(v)] *www.vcf-online.nl,* [(n)] *www.nist.gov*
(-) not found. In ***bold****, odor description of the panel. (*) Calc: Kovats Retention index calculated; Lit: Kovats Retention index from literature*
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4.3 Innovative process combining roasting and dehulling for the valorization of jicaro seeds (*Crescentia alata* K.H.B)

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Abstract

An innovative process combining roasting and conditioning was proposed to promote puffing and facilitate mechanical dehulling of jicaro seeds. Roasting was carried out at three temperatures (120 °C, 140 °C, 160 °C) and during six residence times (80 to 180 s), with a previous seed tempering at 15% moisture content. Parameters monitored as a function of time to optimize roasting operation were the followings: percentage of open seeds, whiteness of cotyledons, and content of two volatile compounds, deemed key jicaro note. The optimal roasting was achieved at 160 ºC for 150 s, with 90% of open seeds. The optimal mechanical dehulling was obtained after the conditioning of roasted seed at 10% of moisture content, with a yield of 75%. Lightness loss followed a zero-order kinetics with an activation energy equal to 103 kJ/mol. Ethyl-2-methylbutyrate and 2,5-dimethylpyrazine increased with the temperature and period of roasting.

Keywords

Jicaro seeds, roasting, kinetic modeling, puffing, dehulling, color L* a* b*

Highlights

- Roasting was optimized and combined with tempering to facilitate mechanical dehulling of seeds.
- Dehydration followed a first-order kinetic and moisture content controlled seeds opening
- Dehulling performance depends on the number of open seeds and their moisture content.
- Lightness loss and aroma were attributed to Maillard reactions with an Ea = 100 kJ.mol-1 .
- Optimal conditions were found to comply technological and seeds quality constraints production.

4.3.1 Introduction

Jicaro (*Crescentia alata* K.H.B.) tree was one of the most culturally valued plant growing in pre-Columbian Mesoamerican civilizations such as the Maya, who worshipped it as sacred (Goetz and Morley, 2003; Ventura, 1996). Jicaro seeds are small (9 mm length, 7 mm width and 2 mm thickness), flat and heart-shaped. They are constituted by two white cotyledons surrounded by a dark-brown, very thin and adherent seed coat (Corrales et al., 2017a). They present also a specific and pleasant aroma. In Nicaragua, El Salvador and Honduras, the whole seeds are roasted and ground up with rice and spices into a dusky fine powder, and then added with milk or water to make a traditional beverage, called horchata, with the pleasant jicaro seed aroma. The nutritional value of the jicaro seed has been clearly demonstrated (Corrales et al., 2017a). This small seed is not only rich in proteins and lipids, just like soybean, but it also has the advantage of not containing anti-nutritional factors such as trypsin inhibitors and alpha-galactosides. In addition, the whole seed with its seed coat contains less phytic acid than soybean (Egounlety and Aworh, 2003) and no tannins (Corrales et al., 2017a). The only disadvantage for a better valorization at the small scale industrial level, should be the presence of a seed coat which is grey or black, fibrous, and totally adherent to the cotyledons, and which may badly affect the color, texture and taste of derived products. The seed coat or hull represents a significant fraction of the seed (23%

of its total weight) as it is the case for other oilseeds (32% for cottonseed and oat, 25% for sunflower seed or 7% for soybean) (Krishna and Chandrasekaran, 2013). Therefore, mechanical dehulling of jicaro seed is a challenge that could open different ways of valorization of the seed to develop new added-value products such as vegetal milk, flour, biscuits, pastries, or snacks, with improved sensory properties.

In the literature, there are many ways to dehull the grains or seeds. For example, in cereals, dehulling may be performed by friction or by abrasion using appropriate equipment. This is the case for sorghum or rice, because of their smooth and round-shaped grain. Regarding wheat, milling is used, because of the presence of a crease in the kernel. Flour or semolina are obtained after crushing the grain between cylinders and separating the fragments of pericarp by sieving. The millers are used to carry out conditioning (tempering) of wheat grain at about 15% moisture for few hours to facilitate the separation of the pericarp which increases in elasticity and plasticity (Glenn and Johnston, 1992).

Dehulling of very small seeds, such as flaxseed or sesame seeds, can be carried out by different mechanical techniques (abrasion, friction, rubbing) (Oomah et al., 1996) but the high lipid content of some oilseeds can represent a limit to its effectiveness. Dehulling can be also performed or facilitated by enzymatic or alkaline chemical treatments (Mridula et al., 2014). Thermal treatments are another effective technique used to facilitate the dehulling of some beans. For instance, soaking soybeans or cowpeas in boiling water for several minutes resulted in an easier removal of the seed out layer. All of these techniques are not really adapted for dehulling the small and flat jicaro seed, enclosed in a black and adherent seed coat.

Roasting, another thermal treatment, was used for sesame decortication (Kaya and Kahyaoglu, 2006; Moharram et al., 1990). This technique is interesting because it allows to develop a very pleasant roasted aroma. In the case of jicaro seed, roasting is already used traditionally in Central America before the preparation of horchata. Actually, it has been shown previously that some aroma compounds, ethyl-2-methylbutyrate and 2,5-dimethylpyrazine, are responsible for the pleasant note of roasted jicaro seeds (Corrales et al., 2017b). However, depending of the conditions used during roasting, quality parameters may decrease, the seed may turn off brown, due to Maillard reactions and off flavors may appear.

Roasting not only produces changes in color, flavor, texture, and structure of the seed, but also causes the dehydration of the seed (Kita and Figiel, 2011). In some cereal grain, such as sorghum, corn, or rice, or in amaranth seed, the textural changes are the result of a sudden evaporation of water caused by higher temperatures of the air, with an increase in the volume of the seed, named puffing. The steam in the outer layers of the kernel increases the pressure causing sometimes the rupture of the hull (Castro-Giráldez et al., 2012; Hoke et al., 2007). Puffing of cereals is particularly linked to the moisture content at the beginning of the roasting process (Maisont and Narkrugsa, 2009; Mariotti et al., 2006; Pardeshi and Chattopadhyay, 2010). For example, puffed sorghum showed an expansion ratio of 5.5 when conditioned at 21% of moisture, while this ratio felt down to only 2.8 with a moisture content of 18% (Sharma et al., 2014). For puffed rice, higher expansion ratio was performed at 10.5% of moisture content (Chandrasekhar and Chattopadhyay, 1988).

All these modifications caused by the roasting, especially the puffing, might be desirable to favor the dehulling of jicaro seeds. The real challenge would be to remove the adherent seed coat while keeping the cotyledons intact and white, and maintaining or enhancing the typical jicaro aroma. To our knowledge, no work was already done on the use of roasting for facilitating the dehulling of a small flat seed with an adherent seed coat such as jicaro seed. However, parameters such as seed conditioning, roasting temperature and roasting time, should be studied to optimize roasting and dehulling efficiency. Kinetic modeling of the main parameters which might influence the optimization of roasting (percentage of seed opening, whiteness, key aroma compounds) will be performed.

The aim of this study was to find the optimal conditions of roasting and dehulling of jicaro seed, while preserving the white color of cotyledon and its typical aroma, for a valorization in new added-value products.

4.3.2 Materials and methods

4.3.2.1 Raw materials

Jicaro seeds (*Crescentia alata*) were obtained from the Pacific region of Nicaragua in November 2013 and November 2014. 100 kg of jicaro seeds were sorted to remove foreign material (e.g., gravel, straw, hard shell fragments), washed, and sun dried at the Universidad Nacional Autonoma de Nicaragua (UNAN), León, Nicaragua.

4.3.2.2 Roasting and opening of jicaro seeds

Different conditioning (tempering) at 10, 12, 15 and 20 % moisture (w/w) were tested before of roasting. The jicaro whole seeds were conditioned in batches of 1 kg at 15 % of moisture for 24 h at room temperature in a closed glass flask and then split in samples of 30 g each.

After 24 h, the 30 g conditioned samples were roasted in a laboratory roaster (Probat- BRZ 2, Emmerich, Germany) that include two attached thermometers 0 – 300 ºC, to indicate the roasting temperatures, each with thermos-sensors and capillaries, two cooling sieves, two complete electrical heating device (drums). Each drum with a diameter of 3.8 cm and electric heating of 850 W, including chaff cyclone, volume flow fan 160 m^3h^{-1}, exhaust air volume flow 50 m^3 h^{-1}.

Heating was performed with an electrical resistance situated under the roaster. Permanent rotation of the cylinder enabled uniform roasting conditions. Air velocity in the roaster was measured with a propeller anemometer Voltcraft BL-30 AN, and the temperature was registered using K-type thermocouples.

Roasting tests were carried out with 30 g jicaro seed samples at 120, 140 and 160 °C for six different roasting times: 80, 100, 120, 140, 160 and 180 s. Roasted seeds were then air-cooled for 5 min. The temperature of the seeds was recorded using a standard K-type thermocouple positioned in the core of the seed sample and connected to an acquisition database (Almemo 2690; Ahlborn, Holzkirchen, Germany). All the roasting tests were conducted in triplicate.

Roasted seeds with an open seed coat, were separated manually from the whole seeds, and then counted to calculate the percentage of open seeds (% OS).

4.3.2.3 Physico-chemical analysis

Moisture content

Moisture content was determined gravimetrically by measuring the weight loss of the jicaro samples. 2.5 g of seed samples were dried at 105 °C until reaching a stable weight, according to NF ISO 6496 French standard (ISO, 1999).

Color measurements

Roasted whole seeds were manually dehulled (complete removal of the seed coat). Then roasted dehulled seeds were finely ground in a grinder (Moulinex AR1105,

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LE NUMERO 1 MONDIAL DU MÉMOIRES

France). The color of ground seeds was measured with the L*a*b* system, using a colorimeter Konica Minolta CR-400 (Sensing, INC., Japan), specified by the Commission Internationale de l'Eclairage (CIE). Tristimulus values give a three-dimensional value for the L*a*b* color space. The L-value represents light–dark spectrum with a range from 0 (black) to 100 (white). Color measurements were performed in triplicate.

Scanning Electron Microscope (SEM)

The surface morphology of the jicaro seed was scanned by a scanning electron microscope (SEM) at 50 mA (S-3700 Hitachi High Technologies, Tokyo, Japan). The samples were coated with an ultra-thin film of gold to make them conductive before analysis by 2 min two times. The scanning electron micrographs were obtained in the Research Center Microscopic Structures at the Costa Rica University.

Extraction and identification of volatile compounds of roasted seeds

1.5 g of ground roasted whole seeds were placed and sealed in 10 mL headspace vials with 1.4 mg internal standard of trans-2-octenal (Sigma-Aldrich, France) and extracted by Headspace Solid Phase Micro Extraction (HS-SPME) (Lebrun et al., 2008). The extraction was carried out at 60 °C with 15 min incubation and 30 min trapping and shaking, using a polydimethylsiloxane / divinylbenzene fiber (PDMS/DVB 65 µm, SUPELCO, Bellefonte, PA, USA). The identification of volatile compounds of roasted seeds was performed by gas chromatography - mass spectrometry according to (Corrales et al., 2017b).

4.3.2.4 Kinetic modeling

To represent the dehydration of roasted jicaro seeds, the semi–theoretical model of Henderson and Pabis, describing an exponential decrease of water content during drying, was chosen (Asiru et al., 2013).

$$M = M_0\, e^{-kt}$$

Where M is the moisture content in % (wet basis) at time t, M_0 is the initial moisture content and k is the dehydration constant (s^{-1}).

The lightness loss followed a zero-order kinetic. Therefore, it was modeled according to:

$$\mathrm{L} = \mathrm{L_o} - \mathrm{kt}$$

where L^*_o is the initial lightness at t_o, and L^* the lightness at time t; k is the rate lightness loss in L units s^{-1}.

Dehydration rate and lightness loss were assumed to be temperature dependent according to the Arrhenius model

$$k_T = k_{ref}\, e^{-\frac{E_a}{R}\left(\frac{1}{T}-\frac{1}{T_{ref}}\right)}$$

Where k_T is the rate constant at the temperature T, k_{ref} is the rate constant at T_{ref} (the reference temperature 413,15 K (140 °C)), E_a the activation energy (J. mol^{-1}), T the temperature (K), and R the gas constant (8.314 J. $mol^{-1}.K^{-1}$).

The kinetic parameters (k, k_{ref}, E_a) were identified by nonlinear regression with a least square minimization procedure using the Excel complement "Solver". This procedure allows a more accurate identification of constants compared to the usual logarithm linearization (Van Boekel, 2008). The uncertainty of the parameters was obtained by the Visual Basic Editor VBE Macro ''Solver Aid" (de Levie, 2001).

4.3.2.5 Mechanical dehulling of the roasted seeds

Conditioning of the roasted seed samples

Jicaro seed samples (10 g) roasted at 160 °C and 160 s, were placed into glass bottles, sprayed with distilled water, covered and returned periodically for a better repartition of water all around the seeds. Water, at room temperature, was added in order to rehydrate the seeds at various moisture contents (7.5, 10, 12.5, and 15 %) and various conditioning periods (1, 2 and 4 hours). The amount of water added was calculated according to the mass balance (equation 4):

$$\text{Water (g)} = w \times \frac{m_d - m_i}{100 - m_d}$$

where w is the sample weight (g); m_d the final moisture content (%); and m_i the initial moisture content (%).

Mechanical dehulling of the conditioned seeds

Dehulling of roasted jicaro seeds was performed using a Satake dehuller (Model THU 35B, Japan). Seeds were distributed, slowly and continuously fed using the 3170 Perten feeder (Perkin Elmer, Japan), between the two rubber rolls of 125 mm rotating in opposite directions and at different speeds, left roll at 1044 rpm and right roll 1888 rpm.

The distance between the rolls was adjusted to the seed size (2 mm thickness). The differential speeds caused a better friction and removed properly the seed coat, driven into a cyclone (air speed 0.86 m s^{-1}) by a ventilation system. The seeds, separated from the seed coats, were recovered in buckets.

Dehulling efficiency

The dehulling efficiency D_{ef} is the amount of cotyledons obtained from the roasted seeds after removing the seed coat. The ratio of cotyledon in a roasted seed is defined as and $R_{\text{coty}} = w_{\text{coty}}/w_{\text{seed}}$, where w_{coty} is the weight of the cotyledon and w_{seed} the total weight of the seed (including the seed coat). This ratio has been calculated previous to the experiment and accounts for 0.77. Then, if w_{in} is the total weight of the roasted seeds introduced in the dehuller, the dehulling efficiency and is defined as follows:

$$D_{ef} = \frac{w_{\text{dehulled}}}{w_{\text{in}} \times R_{\text{coty}}} \times 100$$

where w_{dehulled} is the weight of the dehulled seeds, (which are only composed of cotyledon) and $w_{\text{in}}\ x\ R_{\text{coty}}$ is the weight of the cotyledon introduced into the dehuller.

4.3.3 Results and Discussion

During roasting of jicaro seeds visual changes were observed such as fine cracks and rupture of the seed coat. Scanning electron microscopy revealed a seed coat structure with a regular wrinkled surface (**Figure 4.5 (A)** and **(B))**. The seed coat is strongly adhered to the cotyledons and is constituted mainly by cellulose, hemicellulose and lignin (Corrales et al., 2017a). During roasting, besides the loss of water the seed volume can be increased up to 2 fold (Corrales et al., 2017b). This expansion ratio may induce the rupture of some seed coat. At initial moisture content of jicaro seeds (6.5%), opening started at 130 °C for 4 min of roasting, but cotyledons became dark. At 150 °C only 43 % of seeds opened after 3 min of roasting, and became quickly burnt. At a lower roasting temperature (110°C) no seed opening was observed even during a 10 min period. Therefore, the tempering of the seeds at higher moisture content has been tested. The addition of water to reach 15% moisture content followed by a 24 h rest period, has resulted in a uniform and better swelling of the seeds during roasting at 150°C, causing opening without affecting the white color of cotyledons.

Tempering helped to soften the seed coat, as it is also the case for the wheat pericarp in wheat milling industry (Mabille et al., 2001), and promote a better puffing.

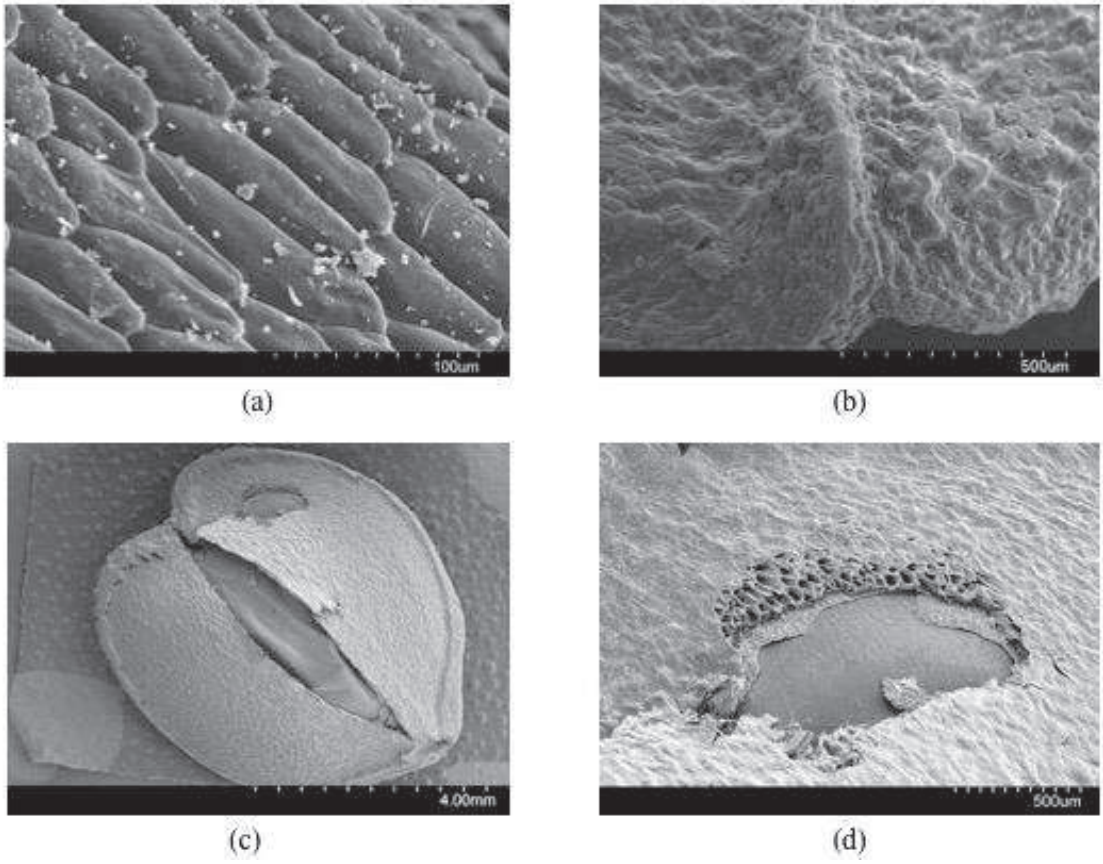

Figure 4.5. *Scanning electron microscopy (SEM) of jicaro seed before and after roasting. (a) and (b) Surface of the seed coat before roasting;(c) Rupture of the seed coat after roasting; (d) Porous and brittle appearance of the seed coat after roasting*

A tempering at 15% moisture was chosen because, at that moisture content, the seed coat softened, became more elastic and less brittle, and the amount of water added was sufficient to maximize the opening of seeds. After tempering initial moisture content of seeds at 15%, different roasting temperatures at 120, 140 and 160 °C were tested and temperature evolution inside jicaro seeds during roasting at these set points are shown in **Figure 4.6 (A)**. The set temperature was achieved in about 20 s inside the flat seed. Heat diffusion inside the seed and time to evaporate free water are responsible for this lag time. Slight variation in the temperature of the seeds could be observed, certainly because of the rotation of the heating cylinder in the roaster, resulting in cycles of residence time of the seeds in contact with the heating cylinder and their fall inside the cylinder. These variations were more important with the increase of temperature.

However, because of a short transient time (20 s) compared to the whole roasting duration (180 s), the thin size of jicaro seeds and their low water content, the heat treatment was deemed to be isothermal. Jicaro seeds are small. Their geometry can be assimilated to an oblate spheroid with a long and small radius of 3 and 1mm respectively leading to a volume to surface ratio of 5 10^{-4}. Assuming a heat transfer coefficient of 15 W/m².K and a thermal conductivity of 0.15 W/m.K (Ashtiani et al., 2014), the resulting Biot number is 0.05 i.e. inferior to 0.1. This means that the internal resistance is negligible and therefore the temperature can be considered uniform within the seed.

Kinetics of jicaro seed dehydration during roasting and their modeling are represented in **Figure 4.6 (B)**. Seeds lost rapidly moisture during the early phases of roasting and then slower when equilibrium was approached. Changes in the seed moisture content were minimal during the roasting interval of 150 to 160 s when the highest amount of free water was released. Dehydration curves followed a typical first order response. Overall, Henderson and Pabi model provided good predictions (with $R^2 = 0.97 - 0.99$). Dehydration rates were 0.0085, 0.0105 and 0.0126 s^{-1} at 120, 140 and 160°C respectively. These values were 20 times higher than rates obtained with the roasting of sesame seeds (Kahyaoglu and Kaya, 2006). This difference might be explained by the different characteristics of the seeds and the different type of roasters (large scale industrial roaster in the case of sesame seeds). However, the activation energy for sesame seeds was similar to the one found for jicaro seeds (13.4 and 14.6 $kJ.mol^{-1}$ respectively) **Table 4.9**.

Table 4.9. Arrhenius parameters of moisture and lightness losses of jicaro seeds roasted at the reference temperature (140°C)

	Order	**T_{ref} (°C)**	**k_{ref} (s^{-1})**	**E_a (kJ/mol)**	**R^2**
Moisture loss	1	140	0.0104 (8.6 10^{-5})*	14.6 (0.7)*	0.99
Lightness loss	0	140	0.0136 (0.0013)*	102.6 (7.6)*	0.98

** Data between brackets represent the standard deviation (n = 3)*

The kinetic of seed opening is presented in **Figure 4.6 (C)**. For each roasting temperature, the kinetic was sigmoid-shaped. More than 80% of open seeds were obtained and longer roasting times were required for lower temperatures. Jicaro seeds suffered a maximum of open seeds when exposed at 160 °C. The percentage of open seeds as a function of seed water content showed that dehydration and popping are

linked **Figure 4.6 (D)**. Interestingly, for all the three roasting temperatures, a 90% opening was obtained at a similar residual water content of 2.5% **Figure 4.6 (D)**. Therefore, we can use the parameters founds of **Table 4.9** to model the jicaro seeds dehydration and calculate the time necessary for reach 2.5% residual moisture content and 90% open seeds, at different roasting temperatures.

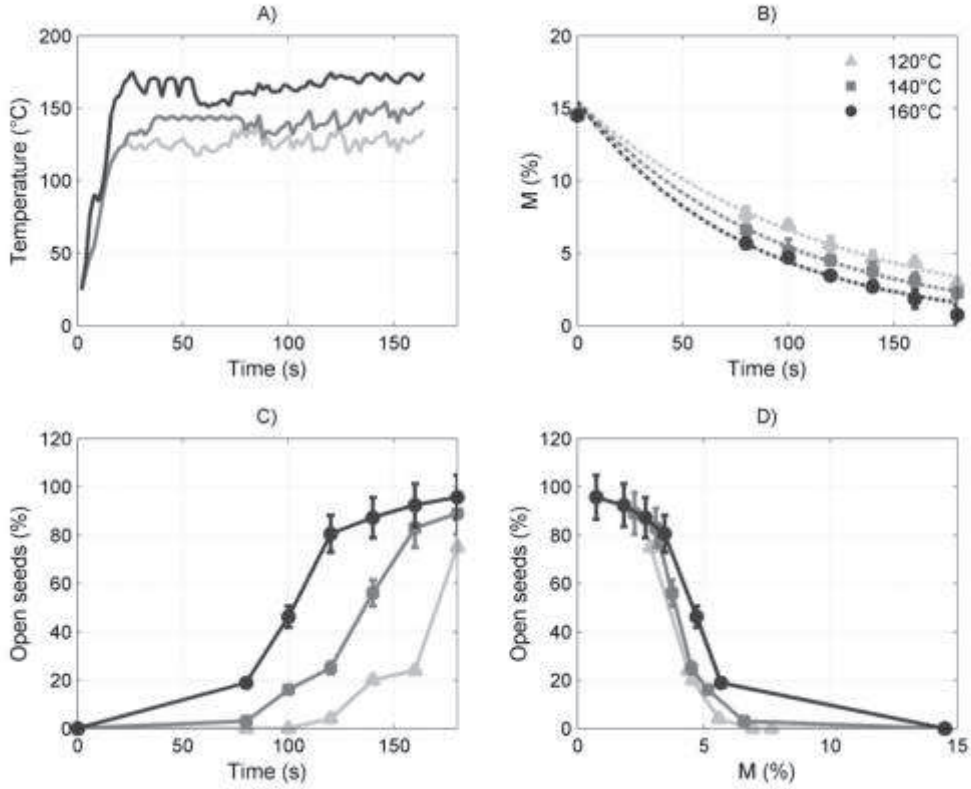

***Figure 4.6**. Kinetics at three roasting temperature (120ºC ▲, 140ºC■ and 160 °C●) of A) seed heating, B) seed moisture loss, C) percentage of open seeds, as a function of roasting time and D) percentage of open seeds, as a function of seed moisture content. Error bars represent the standard deviation (n = 3) and dotted-lines in B) represent water loss modeling.*

The evolution of jicaro seed color during roasting was studied (**Figure 4.7 (a), (b)** and **(c))**. Color parameters (L*, a* and b* value) were measured on manually dehulled seed after roasting at the different temperatures and periods. Before roasting, the initial values of seed color parameters were L*=79.19 ± 0.56; a*=1.24 ± 0.11 and b*= 13.48 ± 0.25. The lightness (L* value) was constant during a certain period of roasting before declining, the higher the roasting temperature, the faster the decrease **Figure 4.7 (a)**. The experimental standard deviations of L* values were quite high, probably because of the important variations of roasting temperatures observed in the heating cylinder **Figure 4.7 (a)**. This induction period was also observed during hazelnut roasting and

was also described as inversely proportional to roasting temperature (Özdemir and Devres, 2000).

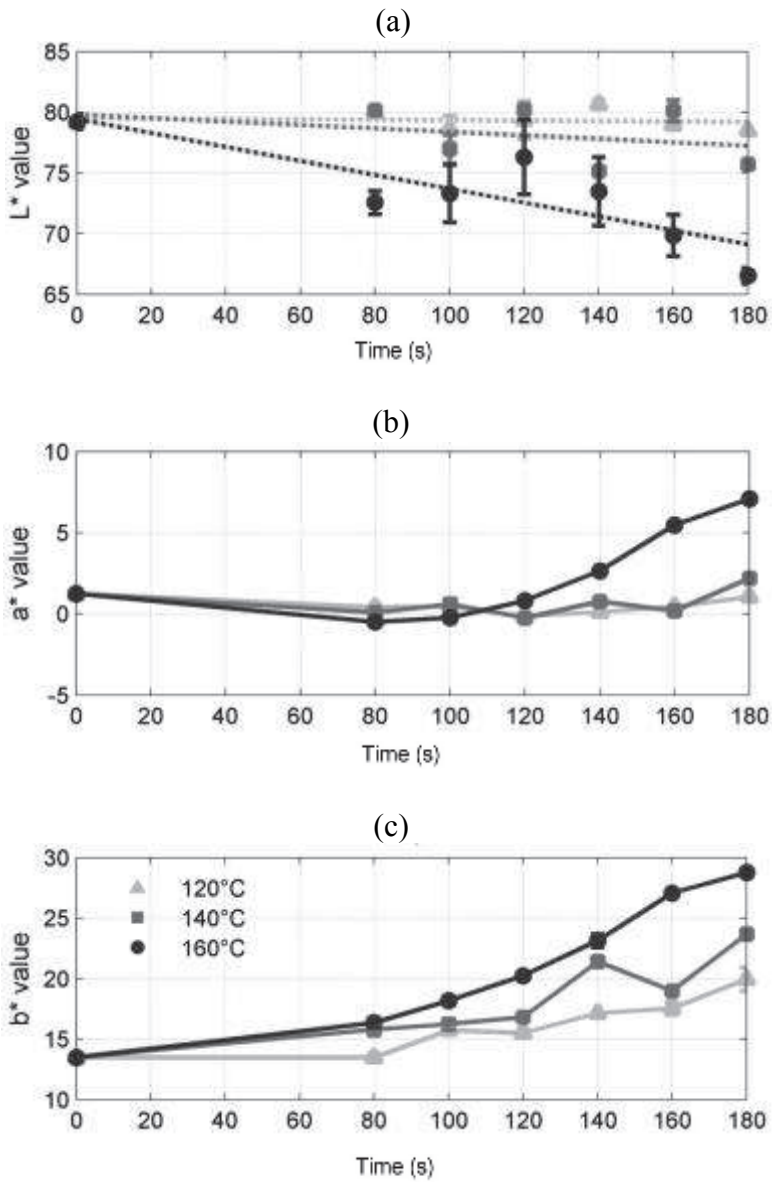

***Figure 4.7**. Kinetics of (a) lightness loss; (b) a *value; (c) b* value as a function of roasting time at three roasting temperatures (120ºC ▲, 140ºC ■ and 160 °C ●). Error bars represent the standard deviation (n = 3) and dotted-lines in A) represent lightness loss modeling*

Contrary to seed lightness, a sharp increase of a* values and b* values were observed at 160 °C (**Figure 4.7 (b)** and **(c))** indicating an increase in the degree of redness (a* value) and yellowness (b* value) with the formation of brown pigments from Maillard reaction. The increase started at 120, 140 and 160°C for a period of 140, 120 and 80 s respectively. The same tendency was observed during roasting of sesame seeds at 120 - 180 °C for 20 - 120 min in a large rotary roasting machine (Kahyaoglu

and Kaya, 2006). However, in our case, a* value and b* value at 160 °C did not exceed 3 and 25 respectively, which allows to qualify the color of cotyledons as white. The roasting process used for jicaro seeds may be defined as a mild roasting process (Corrales et al., 2017b).

The activation energy (E_a) for the lightness loss of roasted jicaro seeds was estimated at 103 kJ mol^{-1}. It is well known that an increase in temperature increases the rate of Maillard browning (Agila and Barringer, 2011). **Figure 4.7 (a)** shows that near 160 s, the product started to darken at 160 ºC (L* value lower than 70), as the rate of moisture removal was decreasing. Therefore, this reaction is more dependent on temperature than popping. Indeed, the activation energy of Maillard reaction was 6 times higher than that of popping. This value of E_a is closer to the typical value of non-enzymatic browning in food (Özdemir and Devres, 2000). These parameters were also used for optimization.

To model lightness loss during hazelnut roasting, Özdemir and Devres, (2000) tested zero-order and a third-order polynomial model. The best fit was logically obtained for the polynomial model. In our case, because of the high standard deviation of L* values, both models gave similar fit results (R^2 =0.7 - 0.9). We therefore chose the simplest zero-order model **Figure 4.7 (a)**. The interest was to also represent the rate constant dependence with temperature according to Arrhenius model **Table 4.9**.

In addition to opened seeds yield, moisture content and color, aroma is also an important parameter to assess. 2,5-dimethylpyrazine and ethyl-2-methylbutyrate have been identified as responsible of the pleasant aroma of roasted jicaro seeds, and were previously assessed by SPME-olfactometry analysis and detection frequency (Corrales et al., 2017b). These aroma compounds must be preserved and eventually enhanced in roasted seeds. The concentration evolution of the two volatile compounds, ethyl-2-methylbutyrate and 2,5-dimethylpyrazine, is presented in **Figure 4.8 (a)** and **(b)** respectively. As the temperature and the roasting time increased, the concentration of 2,5-dimethylpyrazine increased. At 160 °C and 160 s the concentration was 0.09 µg kg^{-1}, which was enough to produce roasted notes, but without altering the whiteness of the seeds. Pyrazines are well-known indicators of the occurrence of Maillard reaction (Ling et al., 2015). They contribute to the desirable roasted flavor of many foods or beverages such as coffee beans (Hashim and Chaveron, 1995; Moon and Shibamoto, 2009). Their

level is directly correlated to the extent of browning that food undergoes as a result of roasting (Koehler et al., 1971).

The ester concentration of roasted jicaro seed increased with the increase of the roasting temperature, particularly at 160°C, and reached a plateau from a roasting time of 80 s for each of the three roasting temperatures. Since the ethyl-2-methylbutyrate is an ester of short chain, it has a high volatility (Liu, 1994). This suggests that a temperature of at least 120 ºC is needed to increase the release of the ester. Moreover, roasting also influences flavor binding due to the unfolding of protein (Preininger, 2005). Roasted jicaro seeds showed changes in the internal microstructure such as protein denaturation (Corrales et al., 2017b), which increased the number of hydrophobic binding sites with the volatile compounds. However, in our mild roasting conditions compared to those carried out on other products (Corrales et al., 2017), temperature and time were sufficient to promote the release of the pleasant roasted aroma of pyrazines, but not too high, to limit the browning of cotyledons by maintaining high lightness values.

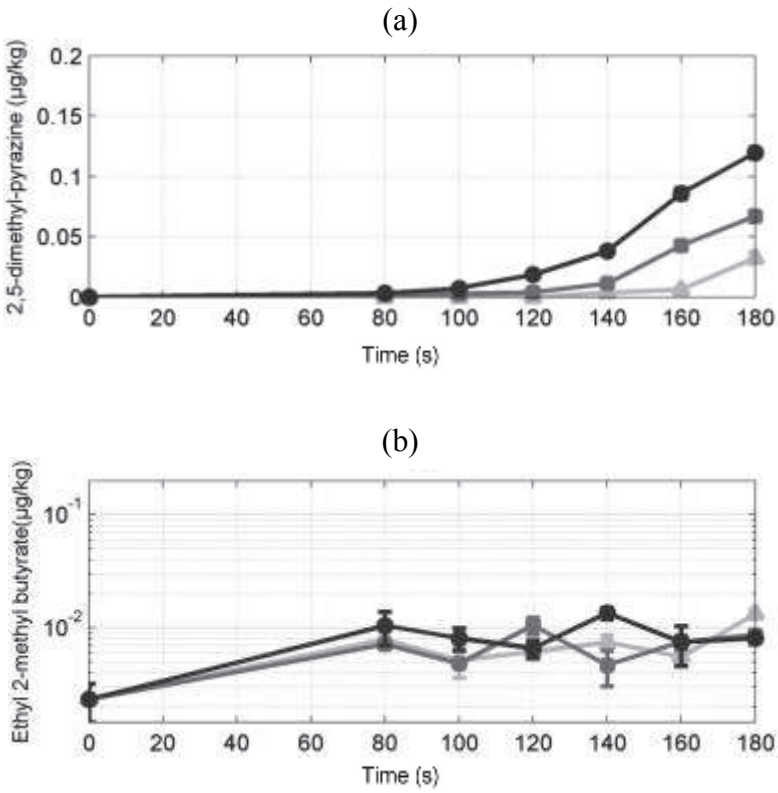

Figure 4.8. *Kinetics of a) 2,5-dimethylpyrazine; b) ethyl- 2-methylbutyrate, as a function of roasting time at three roasting temperatures (120ºC ▲, 140ºC ■ and 160 °C ●). Error bars represent the standard deviation (n = 3).*

As both key aromas increase steadily during roasting, optimization of the operation was done taking into account a minimum of 90% open seeds (i.e. with a residual water content of 2.5 %) and lightness loss of less than 10% (i.e., L ≥ 72). The area that complies with these limitations is represented roughly in **Figure 4.9**. Optimal roasting time should be above the dotted line to obtain a satisfactory puffing for facilitating seed dehulling, and below the solid line to avoid excessive browning.

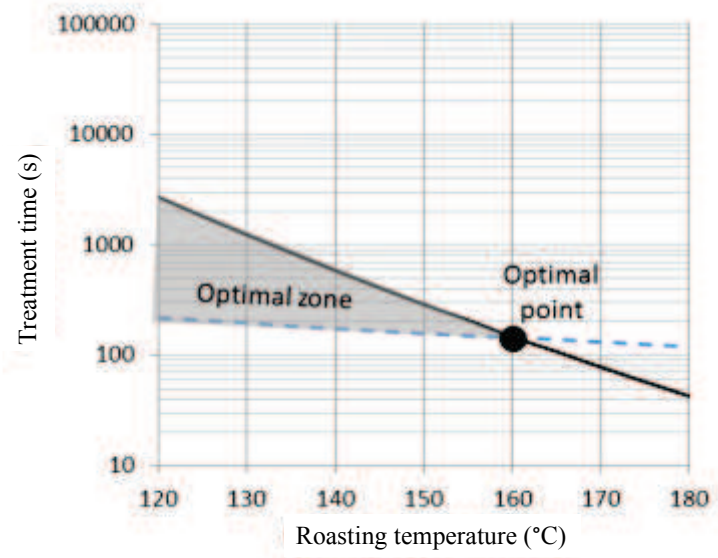

Figure 4.9. *Acceptable operating zone conditions (temperature and time) in gray considering maximum puffing (corresponding to a residual seed moisture content of 2.5% in dotted line) and minimum browning of jicaro seeds (L* value ≥ 72 in plain line), and optimal point (●) maximizing the desirable aroma and minimizing roasting*

Therefore, the optimal conditions minimizing the process time while complying with the constraints of puffing yield and color is 160 °C - 150 s (with a prior conditioning at 15% moisture for 24 h). Furthermore, this high temperature enhanced the production of key aromas compounds generating larger amounts of pyrazines, while maintaining the ethyl-2-methyl butyrate, the key jicaro note. These objectives should satisfy processors of added-value products, both at the economic and at the qualitative levels if the opened seeds can be easily dehulled. After roasting, only open jicaro seeds could be easily dehulled using Satake dehuller. But because of the friable structure of the roasted seed, the cotyledon was totally decomposed to powder between the dehuller rubber rolls which was not desirable. Therefore, a rehumidification step (conditioning) is necessary before dehulling to maintain the integrity of the cotyledons. Dehulling

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efficiency of roasted jicaro seeds as a function of their moisture content after 2h of conditioning, is shown in **Figure 4.10**. Different conditioning times were tested. The better dehulling efficiency (75 %) was reached with water addition of 10 % after 2h of conditioning and stayed stable for longer times thanks to an optimal elasticity of seed coat and hardness of the cotyledon. Conditioning at lower moisture contents maintain the jicaro seeds structure too porous (disintegration of the seeds during dehulling). Conditioning the seeds at higher moisture contents turned the seeds sticky and the seed coat was not properly removed.

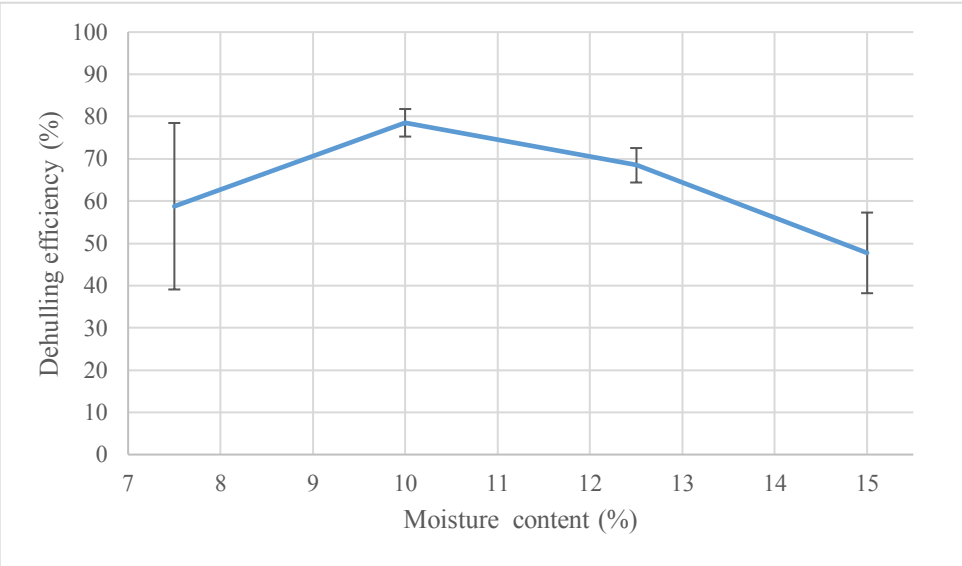

Figure 4.10. *Mechanical dehulling efficiency of roasted jicaro seeds as a function of seed tempering at various moisture content during 2h.*

Finally, the optimal flowsheet and process conditions is presented in Figure 4.11. To obtain white cotyledons of jicaro, two tempering are needed before and after roasting (160°C – 150 s), at 15 and 10 % moisture addition respectively. These three steps allowed to optimize the final dehulling step.

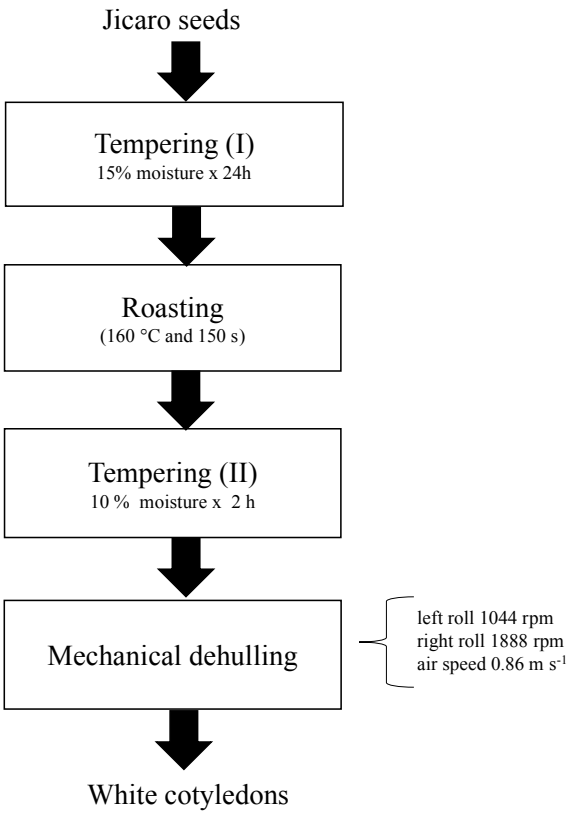

Figure 4.11. Flowsheet of the obtaining of white jicaro cotyledon.

4.3.4 Conclusion

An innovative process was developed for dehulling jicaro seed, a highly nutritious oilseed. The difficulty to overcome was that the seed is very small, flat, with a black seed coat, fibrous, brittle and totally adherent to the white cotyledons. The challenge was to successfully remove the seed coat, while keeping the cotyledons intact and white. Roasting, which is traditionally used in Mesoamerica, has been proposed, after a prior conditioning of the seeds, to cause a phenomenon of puffing, which, thanks to a sudden departure of the water, allowed the opening of the seed coat. Optimum roasting conditions have been determined to obtain an opening of a maximum of seeds, while keeping the seed white and maintaining or enhance the typical aroma of jicaro.

The optimal roasting conditions were 160 ºC -150 s. They enabled 90% of seed opening and preserved the key aroma without affecting the white color of cotyledons. A subsequent conditioning of the roasted seeds to a moisture addition of 10% resulted in an optimal dehulling efficiency of more than 75%. Mechanical dehulling is a fundamental operation to extract the white cotyledons of the jicaro seeds and obtain the

white proteinaceous and oleaginous material from which the jicaro milk would be produced.

Acknowledgments

The present study was supported by PRESANCA II/CSUCA grant no. C-15-2012, the French Cooperation in Central America (Institut Français pour l'Amérique Centrale-IFAC) and the Europea Union Latin America Academic Links. The authors wish to thank Laurent Berthiot for his contribution to the roasting process.

References

References of the article have been removed as they are accessible at the end of the thesis.

4.4 Preliminary study on the elaboration of a vegetal milk from dehulled jicaro seeds

4.4.1 Introduction

The jicaro tree is a natural vegetal that grows spontaneously in the savannah of dry tropical regions in Central America. The tree is resistant to hot climates, fires, floods and sustained droughts due to its pivoting roots. Although there are also some small plantations today it cannot be considered as a cultivated crop.

In some Central American countries, such as Nicaragua, the jicaro has a particular socioeconomic importance. Productive activities linked to the jicaro tree are generally conducted by rural families with limited resources. Their income and food both depend on the sale and consumption of this natural resource. Harvesting of jicaro fruits and processing of jicaro seeds are carried out mostly by women in traditional conditions. They are organized is small cooperatives for the commercialization of the seeds after extraction from the fruit pulp and drying. Alternatively, they roast and grind the seeds, and sell them as a powder. However, this traditional processing requires a hard and long labor because of lack of equipment's to make this process more efficient as described in Section 2.5.

There are few experiences in the commercial exploitation of the jicaro. The Nicaraguan company Reichel SA has a plantation of 370 ha which combines pastures and around 120,000 jicaro trees with a production of 420 tones of fruits per year. Its main activity has focused in the extraction of seeds and their exportation to the neighbor countries, mainly El Salvador. A lesser activity is the production of a flour made of ground jicaro seeds and used for making a beverage named horchata and biscuits. A gourmet liquor is made from the pulp and commercialized as well. The shells are used as combustible. Likewise, this company has developed a biodiesel project using jicaro seeds as raw material.

Other industries of cereal processing, including El Caracol, Sabemas and Rey del Campo, also commercialize jicaro seeds as a powder ready to be used in the preparation of horchata. Some of these brands propose a composite formulation including jicaro powder and other ingredients such as rice, powdered milk and cinnamon. Rice facilitates the grinding of jicaro seeds and brings a lighter color to the resulting milk, which is usually darker than other vegetal milks, because of the presence of black seed

coat. Parmalat S.A., is a company engaged in the production of milk and milk products, has also industrialized since 2008 also a beverage made from ground jicaro seeds added with cow milk, under the brand La Perfecta.

Many vegetable milks or plant-based beverages are produced in many countries. All of them are mainly made from seeds, nuts and cereals which are healthy and attractive nutrients sources. Their composition is shown in **Table 4.10** and **Table 4.11**). In addition to its high nutritional value, soybean has bioactive compounds that make it attractive in the prevention of cholesterol-related diseases (e.g. phytosterols) (Lagarda et al., 2006).

Table 4.10. Composition of various raw oleaginous used for making vegetable milks

	Almond	Chestnut	Hazelnut	Walnut	Coconut	Tigernut	Sesame	RDA
Energy (KCal)	589	225	628	654	354	409	644	2000
Lipids (g)	54.65	5.3	60.75	65.21	33.49	23.74	49.7	<70
MUFA (g)	35.01	0.6	45.65	8.93	1.425	16.47	18.8	
PUFA (g)	12.28	1.3	7.92	47.17	0.366	2.21	21.8	
SFA (g)	4.93	3.2	4.46	6.13	29.698	4.02	7.6	<20
Protein (g)	19.13	4	14.95	15.23	3.33	6.13	17.7	50
Carbohydrates(g)	6.2	39.7	16.70	13.71	15.23	42.54	9.28	270
Sugars (g)	5.3	7.9	4.34	2.61	6.23	n.a.	0.45	
Starch (g)	0.11	31.8	n.a.	2.1	n.a.	29.15	0.4	
Dietary Fiber (g)	8.35	7.1	9.7	6.7	9	17.4	7.9	25
Vitamin A (mcg)	0	0	1	1	0	0	n.a.	800
Vitamin E (mg)	24	1.4	15.03	0.7	0.24	10	n.a.	12
Vitamin C (mg)	n.a.	0	6.3	1.3	3.3	6	0	80
Thiamin (mg)	0.21	0.18	0.643	0.34	0.066	0.23	0.791	1.1
Riboflavin (mg)	0.78	0.1	0.113	0.15	0.02	0.1	0.247	1.4
Niacin (mg)	5.3	n.a.	1.8	1.125	0.54	1.8	4.52	50
Vitamin B6 (mg)	0.11	0.3	0.563	0.537	0.054	n.a.	0.79	200
Biotin (mcg)	64	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.5
Folate (mcg)	70	85	113	98	141	200	45	
Vitamin B12 (mcg)	0	0	0	0	0	0	0	2.5
Pantothenic acid (mg)	0.44	n.a.	n.a.	n.a.	n.a.	n.a.	0.05	6
Sodium (mg)	10.36	10.6	11	2	20	37.63	2.31	<6
Potassium (mg)	767.25	591.6	680	441	356	519.2	468	2000
Calcium (mg)	248.25	47.3	114	98	14	69.54	96	800
Phosphorous (mg)	524.88	87.6	290	346	113	232.23	604	700
Magnesium (mg)	258.13	39	163	158	32	86.88	324	375
Iron (mg)	3.59	1.06	4.7	2.91	2.43	3.41	14.6	14
Zinc (mg)	3.6	0.6	2.45	3.09	1.1	4.19	5.74	10
copper (mg)	1	n.a.	n.a.	n.a.	n.a.	n.a.	1.58	1
Selenium (mcg)	4	n.a.	n.a.	n.a.	n.a.	55	n.a.	
Iodide (mcg)	2	n.a.	n.a.	n.a.	n.a.	n.a.	<5	150
Manganese (mcg)	2.54	n.a.	n.a.	n.a.	n.a.	n.a.	1.23	2

Average values shown are expressed per 100 g of edible part in wet basis; n.a. not available. MUFA: mono unsaturated fatty acids; PUFA: poly unsaturated fatty acids; SFA: saturated fatty acids.
(Bernat et al., 2014)

Table 4.11. Composition of various raw cereals and pseudocereals used for making vegetable milks

	Pseudocereals		Cereals							RDA
	Amaranth	**Quinoa**	**Barley**	**Corn**	**Kamut**	**Millet**	**Oat**	**Rice**	**Spelt**	
Energy (KCal)	371	306	352	365	337	378	401	386	338	2000
Lipids (g)	7.02	5.56	1.16	4,74	2.2	4.22	6.9	2.64	2.43	<70
MUFA (g)	1.685	1.4	0.15	1,25	0.214	0.773	2.2	0.83	0.445	
PUFA (g)	2.778	2.1	0.56	2,16	0.616	2.134	2.5	0.89	1.258	
SFA (g)	1.459	0.5	0.25	0,67	0.192	0.723	1.2	0.52	0.406	<20
Protein (g)	13.56	13.8	9.91	9.42	14.7	11.02	16.9	7.5	14.57	50
Carbohydrates(g)	65.25	49.2	77.7	74.26	70.38	72.85	66.3	81.3	70.19	270
Sugars (g)	1.69	5.92	0.8	0.64	n.a.	n.a.	n.a.	n.a.	n.a.	
Starch (g)	57.27	43.27	n.a.	73.3	n.a.	n.a.	n.a.	72.7	n.a.	
Dietary Fiber (g)	6.7	7.9	15.6	7.3	9.1	8.5	10.6	3	10.7	25
Vitamin A (mcg)	0	0	0	11	1	0	0	0	0	800
Vitamin E (mg)	1.19	0.45	0.2	0.49	0.6	0.05	0.7	0.6	0.79	12
Vitamin C (mg)	4.2	0	0	0	0	0	0	0	0	80
Thiamin (mg)	0.116	0.2	0.19	0.385	0.591	0.421	0.76	0.39	0.364	1.1
Riboflavin (mg)	0.2	0.4	0.114	0.20	0.178	0.29	0.14	0.08	0.113	1.4
Niacin (mg)	0.923	n.a.	4.61	3.64	6.35	4.72	0.96	6.8	6.843	50
Vitamin B6 (mg)	0.591	0.2	0.26	0.62	0.255	0.384	n.a.	0.51	0.23	200
Biotin (mcg)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.5
Folate (mcg)	82	40	23	19	n.a.	85	30	97	26	
Vitamin B12 (mcg)	n.a.	0	0	0	n.a.	0	0	0	n.a.	2.5
Pantothenic acid (mg)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1.35	1.5	n.a.	6
Sodium (mg)	4	61	9	35	6	5	2	6	8	<6
Potassium (mg)	508	780	280	287	446	195	429	223	388	2000
Calcium (mg)	159	79	29	7	24	8	54	21	27	800
Phosphorous (mg)	557	230	221	210	386	285	523	303	401	700
Magnesium (mg)	248	210	79	127	134	114	177	143	136	375
Iron (mg)	7.61	7.8	2.5	2.71	4.41	3.01	4.72	1.7	4.44	14
Zinc (mg)	2.87	3.3	2.13	2.21	3.68	1.68	3.97	1.8	3.28	10
Copper (mg)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.63	0.85	n.a.	1
Selenium (mcg)	n.a.	2	n.a.	n.a.	n.a.	n.a.	n.a.	<51.9	n.a.	
Iodide (mcg)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	6	22	n.a.	150
Manganese (mcg)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2

Average values shown are expressed per 100 g of edible part; n.a. not available.
(Bernat et al., 2014)

A large variety of products, such as milk, butter, creams, cheese and yogurt are derived from these almonds, hazelnut, sesame seeds, etc. All these products are lactose-free, animal protein-free and cholesterol-free.

Many people limit their consumption of dairy products because of lactose intolerance, due to low levels of a digestive enzyme the lactase (EFSA, 2010). About 70 percent of the world population has primary lactase deficiency (Heyman, 2006). In South America, Africa and Asia, over 50 percent of the population are reported to have lactase deficiency, and in some Asian countries this rate reaches almost 100 percent (Lomer et al., 2008). As an alternative, they have the possibility to consume lactase-treated dairy products and milk substitutes made from nuts, seeds and cereals or use oral lactase supplements.

Among the commercialized vegetal milks the most consumed ones in the United States are almond milk, soy milk, coconut milk, cashew milk and oat milk according to the report published by industryACR (2016).

The composition of soy milk is similar to that of cow milk, with about 3.5% protein, 2% fat, 2.9% carbohydrate, and 0.5% ash (Liu, 1997).

Regarding its high nutritional composition and its pleasant aroma and flavor, jicaro seed should be an excellent appropriate raw material for the production of a vegetal milk, as an alternative to other vegetal milks. However, so far, no company producing jicaro-based beverages has made a light colored jicaro beverage. The addition of rice is not sufficient to reduce the dark color of a milk made from whole jicaro seeds. These cited companies have shown that a jicaro milk may have its place on the market and may be manufactured in parallel with other jicaro based products. Moreover, in Central America, governments support small-scale jicaro seed producers to improve food security of rural families.

However, the appearance is a handicap to compete against other whiter vegetal milks. Therefore, a solution should be to produce a jicaro milk not from the whole seeds but from the dehulled seeds, so that the white cotyledons might confer their white color to the milk. The dehulling process described in Section 4.3 will be used to obtain the jicaro dehulled seeds.

In this section, we propose to develop a process for making a vegetal milk integrally from dehulled jicaro seeds and with a light color that would make it competitive with respect to other existing vegetal milks. New scientific information will be provided about the products made traditionally on the basis of empiric knowledge. The process, as well as the product will be compared with other popular vegetal milks.

In the next sections, the preliminary work carried out to produce jicaro milk is presented. A process flowsheet is proposed, which should be optimized in the future. Some preliminary results are presented as well.

4.4.2 Process for making vegetal milks

The process for making vegetable milks is similar for all vegetal sources. The conventional steps include soaking, grinding, water extraction, filtration, homogenization and pathogen removal treatments (Bernat et al., 2014). However, depending on the type of raw material and the characteristics of the final product, there

may be slight differences among them (e.g. enzymatic treatment) (Oste and Lund, 2002).

In the case of soymilk, the process includes soaking and grinding of soybeans in water (see **Figure 4.12**). The water absorption of soybeans during soaking is directly related to the changes in textural characteristics and grinding properties of soybeans (Pan and Tangratanavalee, 2003). Soaking facilitates the grinding and extraction of soluble solids. In some cases, it is necessary to conduct the soaking in boiling water for a better elimination of anti-nutritional factors (El-Adawy, 2002).

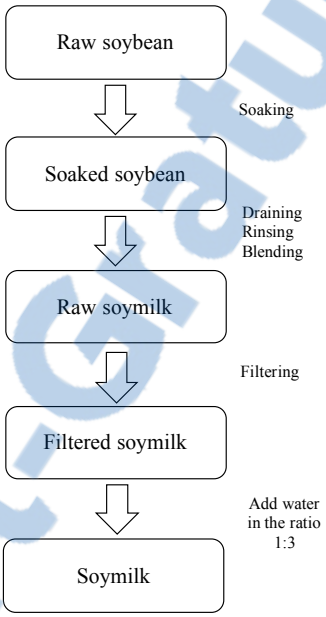

Figure 4.12. *Flowsheet for the production of soymilk*
(Raja et al., 2014)

One of the most important disadvantage during the processing or shelf life of these cereal and nut milks is their low physical stability during storage. A precipitation of non-soluble components such as fibers and a creaming (floating upwards) of fat globules may rapidly occur. An optimal thermal treatment and an under-pressure homogenization were proposed to avoid the separation of phases during storage of a vegetal milk (Valencia-Flores et al., 2013).

Extending the shelf life of the products also requires the removal of the microbiological load. Heat processing is an effective means of achieving this objective (Seow and Gwee, 1997). Ultra-High-Temperature (UHT) is the most employed treatment. It consists of heating the product to high temperature within a short time (e.g. 135ºC for 1-2 s) (Chavan et al., 2011).

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The principle of the homogenization is to reduce the particle size of fat globules in an emulsion. It is carried out by different types of equipment:

- **Conventional homogenizers,** that work at moderate pressures (20-50 MPa) are commonly used in food, pharmaceutical and cosmetic industry to disperse non-miscible phases, stabilize emulsions or prepare products with suitable rheological properties.
- **Conventional homogenizers at high pressures** (100 Mpa). There are several examples of this kind of homogenizers, including GEA Niro Soavi, EmulsiFlex and APV-Gaulin homogenizers. The latter was used in the preliminary assays for the production of jicaro milk. Its principle is the following: the unhomogenized product enters the valve area at high pressure and low velocity. As the product enters the adjustable, closed clearance area between the valve and seat, there is a rapid increase in velocity with the corresponding decrease in pressure. The intense energy release causes turbulence and localized pressure differences, which will tear apart the particles. The homogenized product impinges on the impact ring and exits at a pressure sufficient to move to the next processing stage (**Figure 4.13**). Homogenization at high pressures causes changes in the rheological properties of fluids. Changes in the particle size distribution from bimodal to monomodal are observed as well as a reduction in mean particle diameter (Augusto et al., 2012; Innocente et al., 2009). The effect depends on the pressure applied by the piston pump and the valve design.

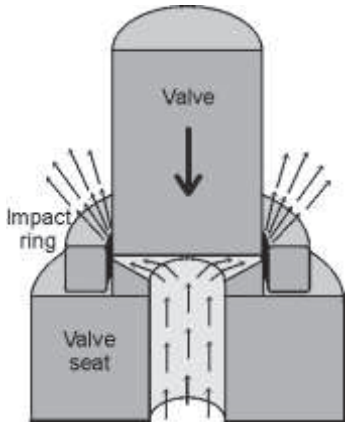

Figure 4.13. *Design of the valve of a conventional homogenizer (APV Gaulin)*

- **Ultra-high-pressure homogenizer (UHPH)** (above 100 MPa) enables achieving a high degree of milk fat dispersion, and improving the microbiological quality of milk. UHPH is considered as an alternative technology to the heat treatments carried out to guarantee milk harnessless, since a marked increase in temperature is noticed during the product homogenization (Cruz et al., 2007; Kiełczewska and Kruk, 2006).

4.4.3 Results and Discussion

4.4.3.1 Process flowsheet of jicaro milk

The successive steps to obtain jicaro milk from whole seeds are shown in **Figure 4.14**. Due to the particular morphology of jicaro seeds, some unit operations have been added or adapted, and differ from the usual process performed for other raw materials (Bernat et al., 2014) The main challenge was to dehull the whole jicaro seeds. Their oily composition, their fibrous and brittle coat and their shape and size were the main difficulties for the dehulling. Nonetheless, with the combination of roasting and tempering, which consisted of the rehydration of the roasted seeds to harden the cotyledon as described in Section 4.3 a good dehulling efficiency was obtained.

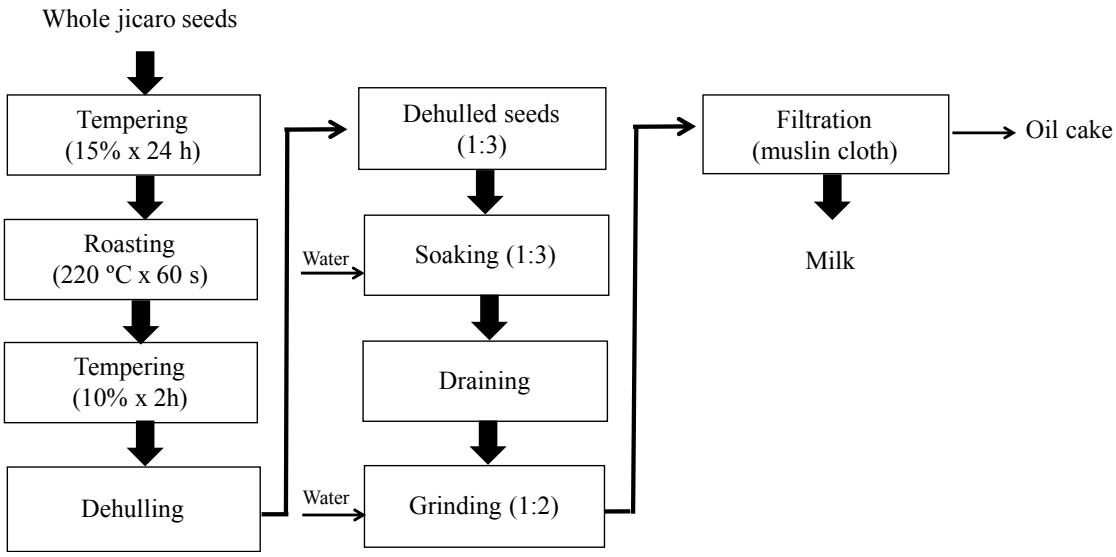

***Figure 4.14**. Flowsheet of the production of jicaro milk*

Soaking time and temperature were considered as the parameters to optimize for a good swelling of the jicaro seeds, which absorbed 47% of their mass in water, with the corresponding increase in volume. The soaking of the seeds facilitates the process of grinding (Hsu, K. H.; Kim, 1983).

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Filtering was carried out with a muslin cloth. Many vegetal milks were produced by using this type of filter (Chaiwanon et al., 2000; Hinds et al., 1997; Rustom et al., 1993). However, this type of filter will present some disadvantages when used at an industrial level, since the volume of production is greater. Indeed, with the usage of a cloth, the pore size widens and larger particles pass through. This is also a problem for the reproducibility of experiments, as it is difficult to keep the hole size constant during the whole assays.

As a result of grinding, an aqueous extraction with particles in suspension was obtained. Therefore, it was necessary to perform a filtering step in order to remove those particles. The residues of the filtration are called oil cake. Milk and cake yields, as well as fat and protein content are directly impacted by this operation.

The jicaro milk obtained with this process is shown in **Figure 4.15**.

Figure 4.15. *Jicaro milk*

4.4.3.2 Effect of soaking temperature and soaking time

The effect of soaking temperature and time is shown in **Table 4.12**. A high variability could be noticed among the results. This may be due to the filtration conditions. As mentioned in the previous section, the pore size of the cloth widens as it is used (at each utilization), so the size of jicaro particles that pass through was not constant during the experiment. Moreover, the manual pressure applied on the cloth filled with jicaro paste, introduces also another source of variability, as it is difficult to maintain it constant.

Through a linear regression, we evaluate, all along the experiments and among the experiments, the effect and statistical significance of the soaking time and temperature.

Soaking temperature and time did not have a statistically significant effect ($p > 0.05$) on the milk and cake yield. **Table 4.12** shows the yield of milk in percentages of dry weight of dehulled jicaro seeds. Global average of milk yield was 32.1% with a range between 27.9% and 36.3%. Cake yield global average was 58.2% with a range from 54.2% to 63.5%.

Fat content of jicaro milk is shown in **Table 4.12**. There was a statistically significant negative relationship between the soaking time of jicaro seed and fat content of jicaro milk ($p = 0.008$) with a decrease of fat content of around 0.32% per soaking hour. During soaking, fat content of the seeds migrates to the surface of the soaking water and several fat droplets were observed floating on the surface of water. This water is discarded afterwards along with these fat droplets. The amount of fat floating in the water was observed to be higher for longer soaking times. This may explain the decrease of seed fat content, and consequently the decrease of milk fat with the increasing soaking time.

Protein content ranged from 3.4% to 4% in all the experiments (see **Table 4.12**). No statistically significant relation was observed with respect to soaking time and temperature.

Table 4.12. Effect of soaking time and temperature on the yield, and fat and protein contents of jicaro milk

Temp (°C)	Time (h)	Milk yield* (%)	Milk dry matter* (%)	Cake yield* (%)	Milk Fat** (%)	Milk Protein** (%)
20	2	31.4	10.8±0.1	62	6.3±0.2	3.8±0.1
60	1	31.9	11.2±0.1	58.1	6.4±0.4	3.8±0.1
60	2	27.9	10.2±0.1	63.5	5.3±0.1	4.0±0.2
60	3	36.3	11.5±0.1	59.3	6.1±0.5	4.2±0.1
70	1	35	11.2±0.1	54.3	6.4±0.2	3.6±0.1
70	2	32	10.3±0.1	54.4	6.0±0.4	3.4±0.1
70	3	28.9	9.2±0.1	57.9	5.0±0.2	3.4±0.1
80	1	32.9	10.4±0.1	58.1	5.5±0.1	3.4±0.1
80	2	33.6	10.8±0.1	54.2	6.1±0.3	3.7±0.5
80	3	30.8	9.6±0	60.2	5.3±0.2	3.4±0.1

(*) Results are expressed in dry weight (d.w) and (**) in wet weight basis (w.w.) ± standard deviations of three replicates

The color of the products plays an important role in the consumer acceptance by the consumers. Therefore, L*, a* and b* values were evaluated for all jicaro milk

produced with different soaking conditions as summarized in **Table 4.13**. Jicaro milk had an opaque white color similar to that of the jicaro cotyledon (L-value of 79.2, a-value of 1.24 and b-value of 13.48).

Milk L and b-values were similar to those of the jicaro cotyledons. However, milk a-values decreased with respect to the cotyledon ones. This is probably because green pigments were liberated during soaking. Indeed, the soaking water turned greenish as it can be appreciated in **Figure 4.16**, an effect which increased with time and temperature. This was confirmed by the statistical analysis, which showed a significant relation ($p < 0.05$) between milk a-values and temperature (decrease of 0.006 per ºC) and time (decrease of 0.097 per hour).

Table 4.13. Effect of soaking time and temperature on color of jicaro milk

Temp (°C)	Time (h)	L*	a*	b*
20	2	79.7±0.2	-0.8±0.0	11.8±0.3
60	1	79.7±0.2	-1.0±0.0	12.8±0.6
60	2	79.0±0.5	-1.3±0.2	13.2±0.3
60	3	79.5±0.2	-1.1±0.0	13.0±0.5
70	1	78.5±0.1	-1.2±0.1	12.6±0.1
70	2	79.6±0.1	-1.2±0.0	13.6±0.0
70	3	78.5±0.1	-1.6±0.1	12.7±0.1
80	1	78.6±0.2	-1.0±0.0	12.6±0.4
80	2	80.2±0.1	-1.5±0.0	12.5±0.3
80	3	78.0±0.1	-1.0±0.0	13.0±0.4

Mean values ± standard deviations of three replicates

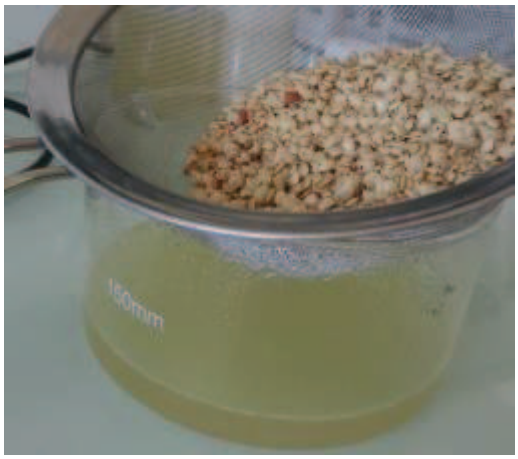

Figure 4.16. *Draining jicaro seeds after soaking*

12 volatiles compounds were identified in jicaro milk. Ethyl-2-methylbutyrate (**Table 4.14**) was identified as the key compound of jicaro aroma as described in Section 4.2. During soaking, a statistically significant (p = 0.0002) negative relation was observed between the soaking temperature and ethyl-2methylbutyrate concentration, with a decrease of 0.0014 mg/kg per ºC (from 0.125 for 2 hour at 20ºC to 0.007 for 2 hour at 70ºC).

Table 4.14. Effect of soaking time and temperature on the concentration of esters and carbonyl compounds in mg/kg (wet basis)

Temp (ºC)	Time (h)	Ethyl-2-methylbutyrate	2-Methylbutyl 2-methylbutyrate	Octanal	Nonanal	Benzaldehyde	Acetophenone
20	2	0.125±0.041	0.190±0.037	0.005±0.003	0.024±0.016	0.153±0.040	0.041±0.004
60	1	0.063±0.005	0.108±0.005	0.006±0.001	0.026±0.006	0.093±0.013	0.034±0.009
60	3	0.066±0.007	0.097±0.010	0.003±0.000	0.011±0.003	0.104±0.010	0.031±0.005
70	1	0.075±0.006	0.091±0.010	0.005±0.001	0.011±0.002	0.132±0.015	0.035±0.010
70	2	0.007±0.003	0.013±0.010	0.000±0.001	0.003±0.004	0.022±0.017	0.008±0.015
70	3	0.084±0.004	0.156±0.008	0.005±0.001	0.016±0.009	0.136±0.009	0.031±0.008
80	1	0.070±0.006	0.162±0.022	0.018±0.001	0.068±0.013	0.146±0.012	0.031±0.013
80	2	0.053±0.007	0.174±0.019	0.009±0.001	0.020±0.005	0.138±0.031	0.035±0.013
80	3	0.049±0.003	0.195±0.025	0.012±0.000	0.044±0.016	0.150±0.012	0.047±0.015

Mean values ± standard deviations of three replicates

Table 4.15. Effect of soaking time and temperature on the concentration of terpenes and aromatic hydrocarbons compounds in mg/kg in wet weight basis

Temp (ºC)	Time (h)	Camphene	Ethylbenzene	Limonene	Styrene	2-methoxyphenol	Phenol
20	2	0.030±0.009	2.026±1.059	0.009±0.002	1.554±0.289	0.014±0.006	0.057±0.012
60	1	0.016±0.001	1.085±0.247	0.003±0.000	0.461±0.102	0.009±0.002	0.038±0.004
60	3	0.017±0.001	1.362±0.265	0.002±0.001	0.530±0.054	0.012±0.002	0.036±0.003
70	1	0.015±0.001	1.311±0.113	0.002±0.001	0.579±0.073	0.009±0.003	0.042±0.005
70	2	0.003±0.001	0.125±0.029	0.001±0.001	0.069±0.088	0.001±0.002	0.004±0.010
70	3	0.002±0.002	1.376±0.178	0.002±0.001	0.697±0.072	0.009±0.001	0.042±0.006
80	1	0.017±0.001	1.348±0.316	0.002±0.001	0.799±0.049	0.009±0.002	0.041±0.007
80	2	0.015±0.002	1.466±0.464	0.007±0.001	0.695±0.106	0.012±0.002	0.046±0.012
80	3	0.016±0.000	1.316±0.231	0.003±0.001	0.636±0.128	0.010±0.001	0.049±0.007

Mean values ± standard deviations of three replicates

4.4.3.3 Effect of the presence of the seed coat in jicaro milk color

Several samples of milk were prepared with a different amount of dehulled seeds. Non dehulled seeds presented the dark seed coat. The results of the color measurements are presented in **Table 4.16**.

Table 4.16. Color values for different percentages of dehulled seeds

Dehulled seeds (%)	L*	a*	b*
100	75.5±0.4	-1.1±0,1	11.2±0.6
95	72.8±0.3	-0.4±0,1	11.7±0.4
90	71.7±0.5	0.1±0,1	10.9±0.5
80	71.4±0.3	0.1±0,1	10.8±0.3

A linear regression was calculated for all the color values with respect to the percentage of dehulled seeds. Statistically significant correlations were found for L* and a*. As the number of non dehulled seeds, and consequently, the amount of seed coat increased, the color of the resulting milk was darker. This confirms our core idea that the dehulling procedure is fundamental for the obtaining of a white milk that is competitive with other vegetal milks.

4.4.3.4 Rheological and particle size measurements of jicaro milk

Figure 4.17. Illustrates the apparent viscosity of jicaro milk and of two commercial milks: soy milk and almond milk, brands CASINO and FJORG respectively, bought in the supermarket. Both jicaro milk and soy milk had a similar Newtonian flow behavior: the viscosity did not change with increasing shear rate. However, the viscosity of almond milk showed a non-Newtonian flow behavior. The viscosity of jicaro milk and soy milk were in the range of 1.8 to 4.5, and 1.9 to 3.8 mPa·s respectively, which can be considered as relatively low values for protein–lipid emulsions (Atarés et al., 2010). Jicaro milk was produced after an aqueous extraction of the seeds which are free of starch. In contrast, the viscosity of almond milk ranged between 3.2 to 43 mPa·s. The different behavior of the almond milk might be due to the presence of starch (3%) and a thickener (i.e. carragenans), indicated on the packaging. These stabilizing agents cause an increase in the physical viscosity of the beverages (Akkarachaneeyakorn and Tinrat, 2015).

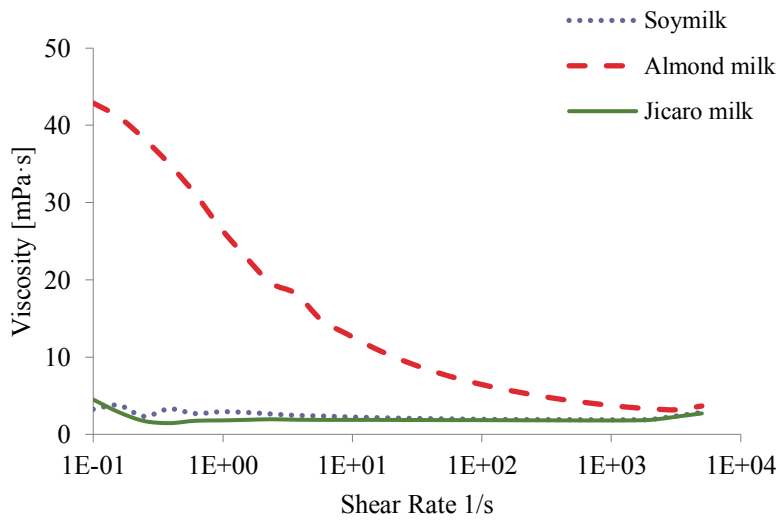

Figure 4.17. *Apparent viscosity of jicaro milk, soy milk and almond milk*

The particle size distribution of jicaro milk produced from seeds soaked in various conditions is shown in **Figure 4.18**. All jicaro milks showed a multimodal particle size distribution with three maxima occurring close to 0.5, 1.5 and 8.6 µm. Soaking time and temperature did not have any effect on the distribution sizes of the milk particles sizes.

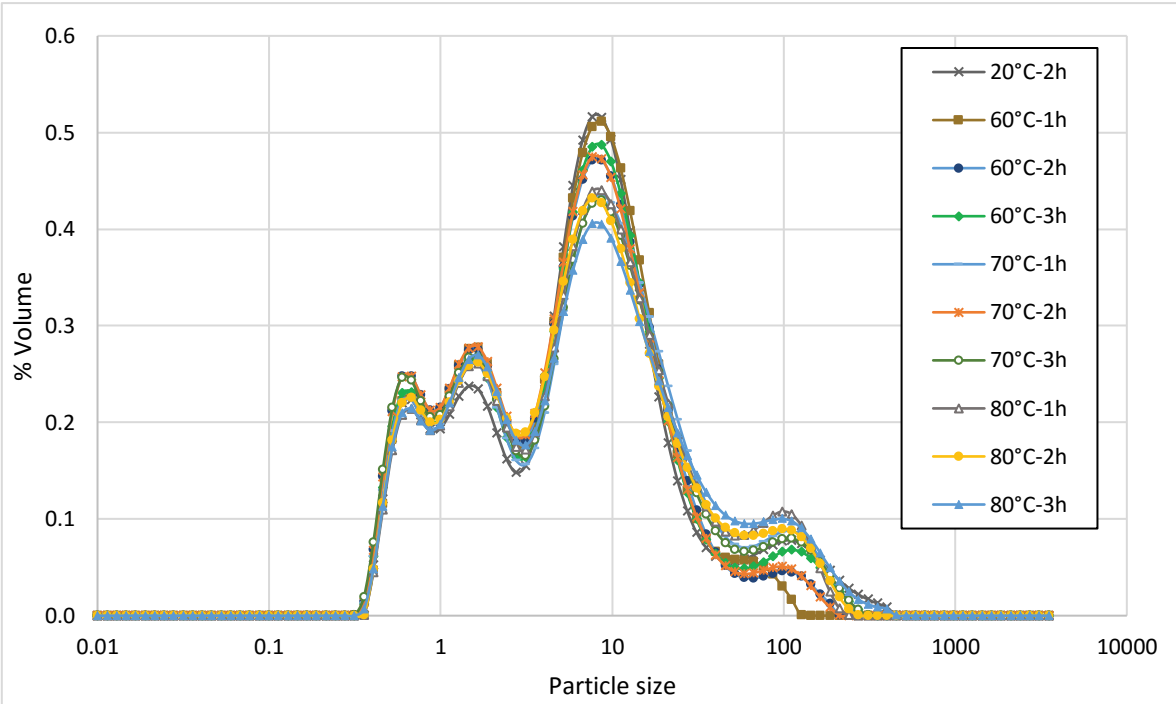

Figure 4.18. *Particle size distribution for different treatments of soaking*

Homogenization reduced particle size distribution as shown in **Figure 4.19**. When the pressure reached 75 MPa, a bimodal size distribution of milk particles was observed, with two maxima at 0.5 and 0.8 µm respectively, producing a more stable emulsion.

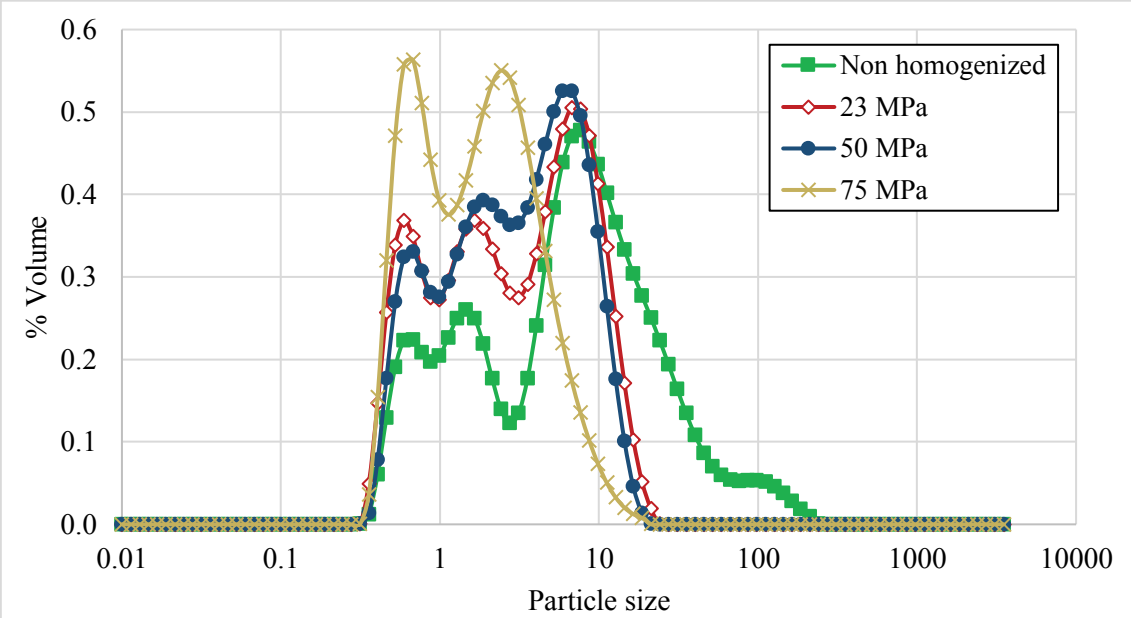

Figure 4.19. *Particle size distribution of jicaro milk after homogenization under various pressures*

In addition, homogenization caused a lighter color in the milk **Table 4.17**. The reduction of the fat globules allowed to reflect the light making a whiter milk. As the pressure augmented, fat globules were smaller and, as a consequence, the milk color was lighter than the original one. This has been also observed in other milks: for instance, in the case of peanuts beverage a whiter and better textured beverage was produced when homogenized at 20 MPa (Rubico et al., 1987).

Table 4.17. Effect of homogenization on the jicaro milk color

Pressure MPa	L*	a*	b*
Control	77.6 ± 0.84	-0.27 ± 0.03	12.1 ± 0.3
23	80.3 ± 0.80	-0.31 ± 0.06	11.7 ± 0.2
50	80.6 ± 0.54	-0.42 ± 0.04	11.4 ± 0.2
75	79.9 ± 0.26	-2.05 ± 0.03	4.8 ± 0.3
Soy milk	79.0 ± 0.06	-4.7 ± 0.07	10.2 ± 0.14
Almond milk	78.7 ± 0.05	-1.4 ± 0.04	2.2 ± 0.12

Mean values ± standard deviation

The distributions of particles of jicaro milk obtained at 60 ºC during 2 hours of soaking and not homogenized, soy milk and almond milk are shown in **Figure 4.20**. Particle size distribution curves of jicaro, soy and almond milk showed a polydisperse distribution. Larger particles were found for almond and soymilk (with peaks around 50 and 80 µm respectively). Soymilk had also a peak of smaller particles (at about 0.5 µm)

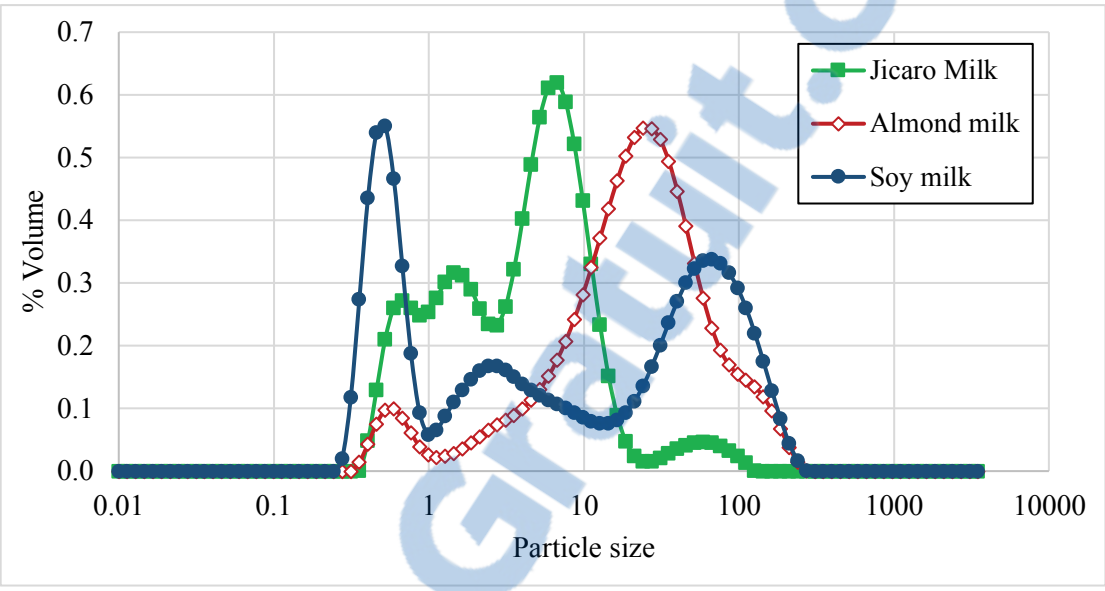

Figure 4.20. *Comparison of the particle size distribution for jicaro, almond and soy milks*

4.4.3.5 Physico-chemical composition of jicaro milk

A partial physico-chemical composition of the jicaro milk, soymilk and jicaro cake is presented in **Table 4.18**. Protein and lipid content of jicaro milk exceeds that of soymilk. Jicaro milk is a beverage of higher nutritional value.

Gamma-tocopherol is the form of vitamin E, which was found in higher concentration (**Table 4.18**). Gamma-tocopherol is the most abundant isomer in oilseeds the content (Speek et al., 1985). In jicaro milk, the gamma-tocopherol content was of 16.1 mg/kg. Beta and delta-tocopherols jointly content was of 1.4 mg/kg. These two isomers could not be identified separately. Alpha-tocopherol, which has the greatest vitamin E potency for humans was not detected in the samples. But depending of their nature, they can differ in their antioxidant capacity. In particular, gamma-tocopherol has the ability to protect against free radicals based on nitrogen, unlike alpha-tocopherol which does not have it (Jiang et al., 2001).

In soybean the contents of tocopherol vary depending on the variety: ranging from 32 to 130 mg/kg for gamma-tocopherol, from traces to 15.3 mg/kg for beta-tocopherol,

7 to 55 mg/kg for delta-tocopherols, and from 15 to 20 mg/kg for alpha-tocopherol (Lee et al., 2015).

After filtration, there remained a residue called jicaro cake which is rich in protein, lipid and gamma-tocopherol. Therefore, this residue could be used as raw material for making other food products such as cookies, bread, meal.

Table 4.18. Physico-chemical composition of jicaro milk and cake

Component	Jicaro milk**	Soymilk**	Jicaro cake*
Protein (g/100g)	3.6 ± 0.1	2.9 ± 0.1	42.2 ± 0.4
Lipid (g/100g)	5.3 ± 0.1	1.4 ± 0.1	43.2 ± 0.8
Ash (g/100g)	0.4 ± 0.01	0.7 ± 0.1	5.2 ± 0.8
Vitamin E (mg/kg)			
Gamma-tocopherol (mg/kg)	16.1 ± 4.1	n.a	69.6±9.4
Beta/delta-tocopherol (mg/kg)	1.4 ± 0.3	n.a	7.5±2.0
Alpha-tocopherol (mg/kg)	n.f	1.2[l]	n.f

(*) Results are expressed in dry weight (d.w) and (**) in wet weight basis (w.w.) ± standard deviations of three replicates. n.f.: not found; n.a.: data not available; .l: from the literature (USDA, 2017)

4.4.3.6 Confocal laser scanning microscopy of jicaro milk

The observation of jicaro milk using confocal laser scanning microscopy revealed small oil bodies (in red) and protein bodies (in green). Oil bodies are spherical (**Figure 4.21**). In the intact cotyledon cells, oil bodies showed a regular structure with spherical proteins bodies (PB) surrounded by much smaller spherical oil bodies (OB) as presented in Section 4.2. As the soybean, jicaro seeds contain protein and oil stored in the cotyledon tissue in organelles called protein bodies and oil bodies (also called oleosomes). Grinding caused the disruption of the cell wall which released oil bodies and protein bodies into the aqueous medium for diffusion. It is known that when the cells are broken, the protein content is released from the interior of the cells (Campbell et al., 2011). After grinding, the product contained abundant proteinaceous material, as shown in the **Figure 4.21**. Also, the microscopic observation showed the coalescence between the oil bodies of small size giving rise to larger fat droplets.

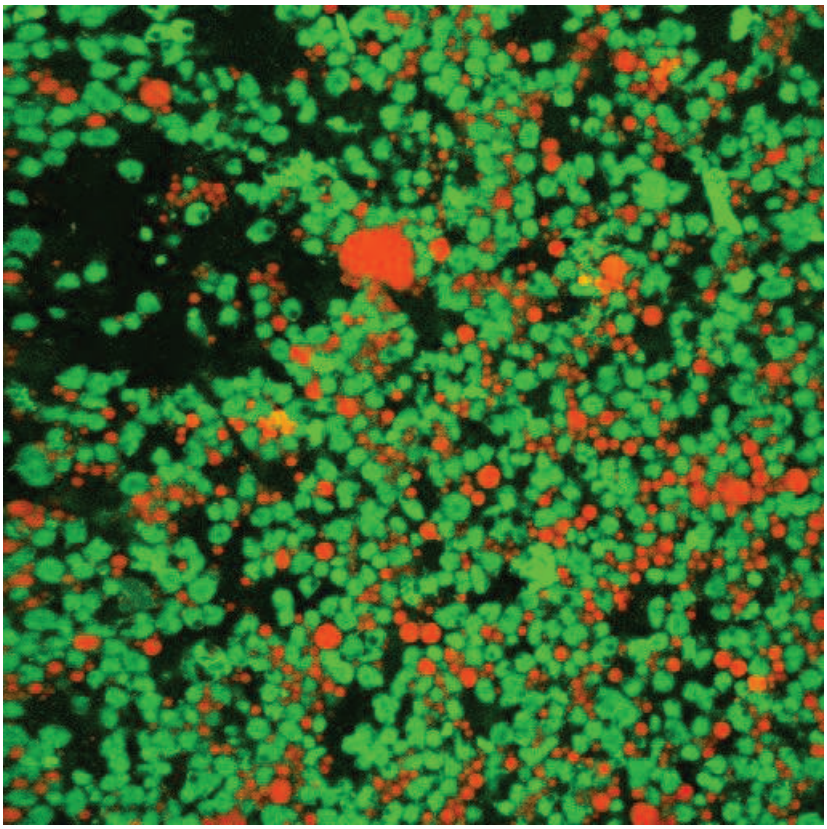

Figure 4.21. *Confocal laser scanning microscopy of jicaro milk (in green protein body and in red oil bodies).*

4.4.3.7 Suspension stability of jicaro milk

A gravitational separation of the fat globules was observed in jicaro milk after some rest time. Jicaro milk, in the same way as other vegetal milks, is an emulsion of oil-in-water (O/W). After a period of storage, lipids which have a lower density than water, float on the surface. This phenomenon is known as creaming. Other denser particles such as fibers and non-soluble proteins dropped to the bottom because of a sedimentation phenomenon. Homogenization reduced the size of fat globules: after 24 h of storage, creaming was reduced from 9% to 3% when a homogenization was performed (see **Table 4.19**). However, sediments augmented. These observations indicate a low stability of jicaro milk. Therefore, it is necessary to investigate the nature of those sediments to improve the stability of suspension.

Creaming and sedimentation are two of the phenomena that appear in breakdown processes in emulsions (O/W) (Tadros, 2013). In some cases, it is necessary to use stabilizer agents to improve the stability of emulsion (Akkarachaneeyakorn and Tinrat,

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LE NUMERO 1 MONDIAL DU MÉMOIRES

2015). For instance, in the case of almond beverage the addition of emulsifying agents is necessary to improve the physical stability of the emulsion (Valencia-Flores et al., 2013).

Table 4.19. Stability of jicaro milk suspension after 24 h of storage

Homogenization pressure (MPa)	Stability of suspension	
	Creaming (%)	Sedimentation (%)
Control	9	16
23	4	20
50	3	21
75	3	23

4.4.3.8 Clinical study with human volunteers

Glycemic response

The results of postprandial glycemic response are show in **Figure 4.22**. The glycemic index (GI) is the result of dividing the area under the glycemic response curve of the jicaro milk by the area under the glycemic response curve of a white bread. The lowest values were obtained for jicaro milk. These differences could be explained by the carbohydrate composition of jicaro seeds, which is low in sugars and does not contain starch as it is described in Section 4.1. Plasma glucose concentration showed a maximum at approximately 60 min after ingestion of white bread (standard food) reaching a value of 124 mg/dl, then a decrease is observed to return to preprandial levels within 2-3h. Both jicaro milk and soymilk reached this peak in half an hour. Jicaro milk had a glycemic index of 11, even lower than that of soymilk, which was 30. Therefore, jicaro milk would represent an alternative in the diet of diabetic people. This is consistent with some studies which indicate that the regular consumption of oleaginous seeds is associated with a reduced risk of fatal ischemia, cardiac diseases, and type-2 diabetes (Esfahlan et al., 2010; Ritter and Savage, 2007; Yada et al., 2011).

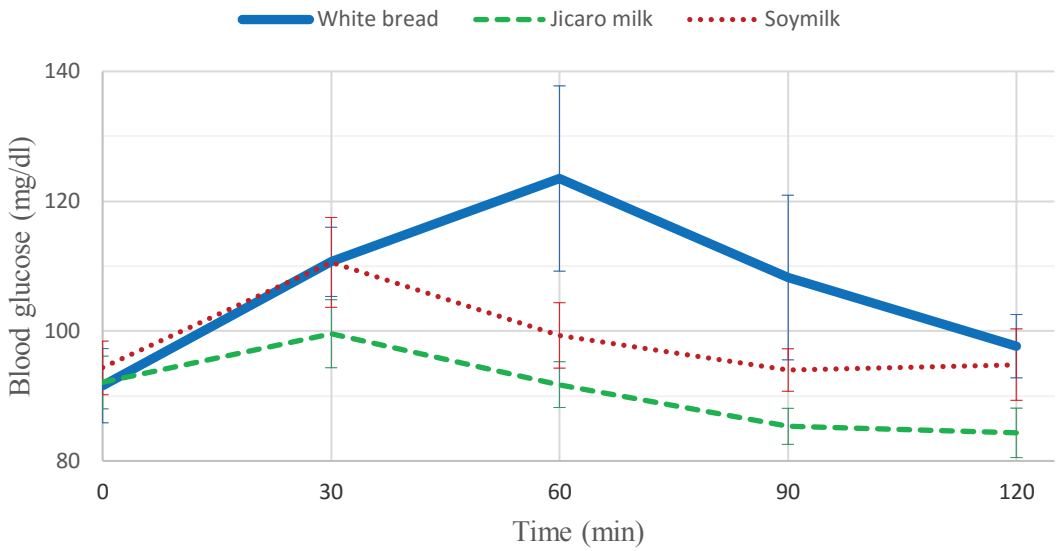

Figure 4.22. *Glycemic response as a function of time, after the intake of jicaro milk, soy milk and bread*

Research of urinary biomarkers of jicaro milk consumption

A clinical study was conducted in our facilities to 8 volunteers to study the urinary biomarkers found after jicaro milk consumption. The study was divided into different phases as described in Section 3.10.2, which included diets with and without the consumptions of jicaro milk. Urine samples were retrieved on the first two days and in the seventh day after five days of consumption of jicaro milk. Comparison between samples collected on day 1 and 2 corresponds to the acute nutritional intervention and comparison between 0-24 h urine sample for day 1 and 7 aimed at evidencing the impact of short term consumption of jicaro milk. The metabolomic study on urine samples was performed combining analysis by UPLC-DAD / ESI^{+}-Q-Tof-MS, extraction of data and subsequent statistical analysis.

For this study, 1779 ions were detected in urine and relative quantification was assessed for all ions in all the samples that were analyzed in random order. The comparison of the metabolome of urine samples for the acute nutritional intervention (days 1 and 2) showed significant differences while short term intervention (days 1 and 7) did not produce significant differences. This can be due to the fact that the nutritional intervention was relatively short, but also because jicaro milk is essentially composed of proteins and lipids, with a relatively low amount of phenolics or other compounds that could be specific of jicaro. The result showed that after 5 days of jicaro milk

consumption urine metabolome came back quickly to the same equilibrium than the day before the nutritional intervention, evidencing no detectable adverse effects.

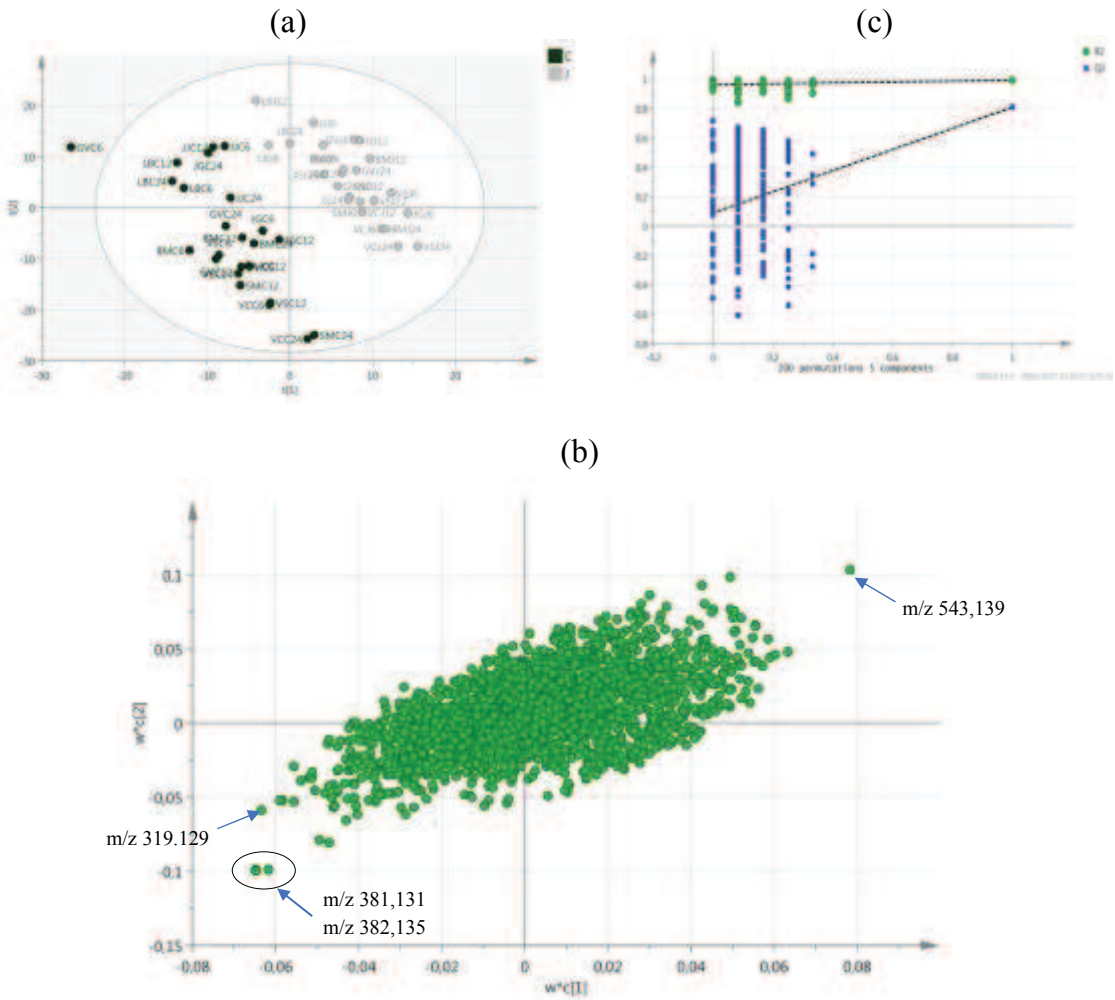

***Figure 4.23**. Score-plot (a), loading plot (b) and results of permutation test (c) of the OSC-PLS-DA model for the comparison of urine samples collected between 0 and 24 h after consumption of 420 ml of jicaro milk (CV-ANOVA=2.10^{-4}, R^2=0.993 Q^2=0.809, R^2 intercept (0.0, 0.963) and Q^2 intercept (0.0, 0.0887) for permutation test with n=200.*

Nonetheless, during the acute nutritional intervention, significant changes could be noticed in the urine metabolome of volunteers. The results of the model that described this acute nutritional intervention are presented in **Figure 4.23**. They show the score plot and loading plot of the orthogonal signal correction for partial least square discriminant analysis (OSC-PLS-DA) model that was deemed statistically robust (CV-ANOVA = 2.10^{-4}, R^2 = 0.993 Q^2 = 0.809, R^2 intercept (0.0, 0,963) and Q^2 intercept (0.0, 0,0887) for permutation test with n = 200). On the basis of this model, it was possible to screen only six ions that had a VIP superior to 2 and a $p < 0.05$. These ions represented

in **Table 4.20** and **Figure 4.23 (b),** appear to be statistically discriminant between urine metabolome of volunteers who consumed a control beverage and the jicaro milk.

This is a very short list of discriminant ions, but as stated before, jicaro milk is essentially composed of proteins and lipids. However, the most discriminant ion (VIP=2.99 and p-value = 8.3 10^{-3}) is m/z 543,152 which increased considerably in urine collected after ingestion of jicaro milk (**Figure 4.24**). In the same cluster, at retention time 0.8 min, a second ion m/z 527,157 was found also to be discriminant, and mass differences between ions shows that m/z 543,152 is $[M+K]^+$ and m/z 527,157 is $[M+Na]^+$. The m/z 505.1763 [M+H] was not detected but it is often the case for ions with abundant Na and K adducts produced at the ionization source. A research in METLLIN and HMDB databases shows that m/z 505.1763 [M+H] may correspond to an oligosaccharide of three simple sugars. This hypothesis is compatible with the high polarity corresponding to the low retention time observed during reverse-phase chromatography (0.8 min). A MS/MS fractionation of this ion produced a main fragment at m/z 365,1087 $[M+Na]^+$ which could correspond to sucrose (m/z 365,1054 $[M+Na]^+$). Therefore, when putting together all these clues, it can be deduced that this ion may correspond to a sucrosyl-oligosaccharides. An extensive bibliographical research shows that sucrosyl-oligosaccharides are actually present in seeds of some plants, and a specific one, planteose (6_F-galactosylsucrose) has been found to be concentrated in the seeds of the family of Bignociaceaes (Brayant et al., 1993; Jukes and Lewis, 1974). This compound may have escaped detection by the regular physico-chemical analysis implemented even though it is similar to raffinose. Planteose ($C_{18}H_{32}O_{16}$ m/z 505.1763 [M+H] or m/z 527.1583 [M+Na]) appears along with sucrose in mature seeds and it is probably a storage carbohydrate utilized for seed germination (Wakabayashi et al., 2015). No information has been reported on its bioavailability, but like most oligosaccharides it could not be metabolized to simple sugars after ingestion. In this case, according to its conformation, it could have either an anti-nutritional effect at high concentration or a beneficial effect on microbiota promoting growth of bifidobacteria for instance. The presence of this oligosaccharide may also explain the low glycemic index observed previously because of competition with simple sugars for site of absorption.

Table 4.20. List of discriminant ions ($p < 0.05$, VIP > 1.5) aroused by the OSC-PLS-DA model for the acute nutritional intervention with jicaro milk.

Tentative identification	Molecular formula	Theoretical m/z $[M+H]^+$	Mass (ppm)	Cluster of ions	Adduct	ANOVA p-value	VIP value	RT (min)	Ratio (before / after)	Principal fragments MS/MS (m/z)
Trisaccharide (Planteose)	$C_{18}H_{32}O_{16}$	505.1690	3	**P543.139** (1)	[M+K]	8.3E-3	2.99	0.8	8.5 (2/23)	365.1087 381.0805
				P527.157 (12)	[M+Na]	5.0E-2	1.91		2.4 (15/37)	
Unknown				**P381.131** (2)		7.40E-3	2.6	13.1	0.5 (165/88)	
				p382.135 (3)		5.59E-3	2.59		0.5 (40/20)	
				p397.106 (4)		4.77E-2	2.44		0.5 (14/8)	
Unknown				P319.129 (5)		1.16E-2	2.24	9.26	0.3 (12/3)	

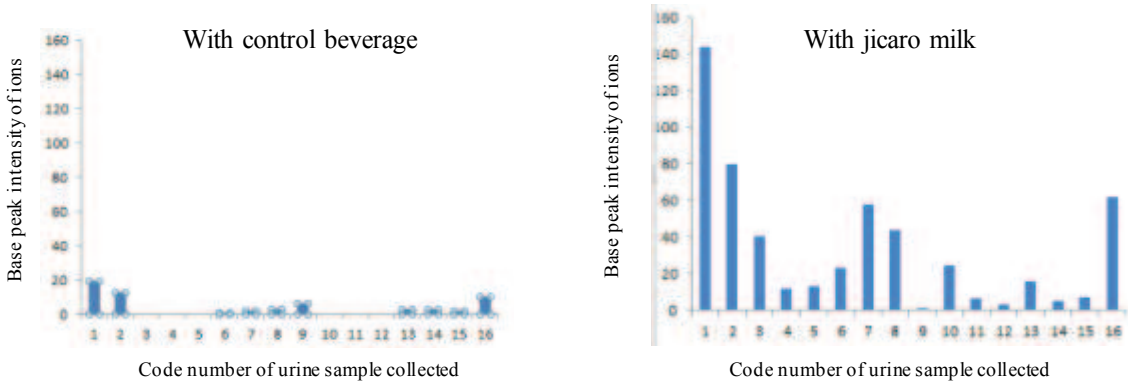

***Figure 4.24**. Histogram of m/z 543,139 [M+K] in urine samples collected after ingestion of a control beverage and jicaro milk.*

For the other discriminant ions m/z 381.131 at 13.1 min and m/z 319.129 at 9.26 min, it was not possible to construct a reasonable hypothesis about a tentative identity. These compounds were present in the urine of volunteers before ingestion and were slightly reduced after ingestion of jicaro milk. The mass of these ions was not reported in the database and they change could be due to the dietary changes that were required for the study. It is important to recall that volunteers received the same diet for control and jicaro milk but residual compounds from their previous diet may have persisted. Therefore, hypothesis or comments on ions that are reduced during the nutritional intervention are often more suspicious than for ions that are considerably increased and be more easily attributed to the food product tested. The hypothesis on the urinary excretion of a sucrosyl-oligosaccharide allowed highlighting an unexpected outcome that was not evidenced during the classical physico-chemical analysis of jicaro seed. A specific method of analysis must be set up to confirm the identity of this tri-saccharide (Wakabayashi et al., 2015), but whatever the identity, it could be of interest for potential health effects.

4.4.4 Conclusion

For the first time an innovative technological process was conducted at laboratory level to produce jicaro milk. The jicaro seed, like other oleaginous, is a good source of protein and lipids for the production of a vegetal milk. The developed process gave a jicaro milk with a higher nutritional value than soymilk. Jicaro milk was obtained by grinding the dehulled seeds with water, according to the processes described above, resulting in an aqueous emulsion.

Experimental results showed that it was possible to obtain a jicaro milk with smaller sizes of particles after homogenization. The obtained emulsion had problems of stability due to the high fat content of the seeds. Therefore, an optimization of the process should be conducted to improve the milk stability.

This work demonstrates the potential of jicaro seed as a raw material with high nutritional content for the production of a vegetal milk. This responds to the need to increase the utilization of nutritious, functional and competitive foods, using native raw materials. Additionally, its typical pleasant aroma, white color and low glycemic index make it an attractive vegetal milk.

In addition, each step in the jicaro seed production chain has a cleaner and environmentally friendly production approach. Moreover, all parts of the fruit of the jicaro can be used, thus minimizing the residues that can be generated.

Chapter 5

General discussion

5.1 Physico-chemical properties of jicaro seeds

The first part of this thesis focuses on the physico-chemical composition of the jicaro seed. The characterization has been performed on three different types of samples: the whole seed as it can be found in the market, the cotyledon and the seed coat.

The principal nutritional components found in the whole seed are proteins, lipids and fiber (33.4%, 33.8% and 23.6%, respectively). The lipid content was found to be comparable to that of other oilseeds such as linseed, cottonseed or safflower seed (McKevith, 1985), and higher than the lipid content in the soybean. With these characteristics, we can consider the jicaro seed as an oleaginous (oilseed).

The protein content (43.6% d.b.) in the cotyledons is comparable with the value found in the dehulled soybean (42%). Its amino acids profile is also similar to that of the dehulled soybean, which is considered a source of high quality protein for human food. This results are consistent with previous studies that demonstrated the good Protein Efficiency Ratio (PER) of the jicaro seeds (Bressani, 1963). Moreover, the proteomic analysis showed that the dominant proteins are of low molecular weight, mainly albumins, which have a good solubility. This property is very convenient for the

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manufacture of a vegetal milk, as the protein content can be extracted without the need of any chemical treatment. This reflected in the high protein content of the jicaro milk, as it was described in Section 4.4.3.

As opposed to soybeans, antinutritional factors content is negligible: the only traces found were for trypsin inhibitors (<0.1 TIU/mg) and alpha-galactosides (stachyose, raffinose: 0.14 and <0.1 % d.b. respectively).

On the other hand, the absence of starch in the composition seed is consistent with other dicotyledonous seeds (e.g. soybean, cottonseed or flaxseed) that during their maturation consume all the starch reserves (Saldivar et al., 2011). This result is consistent with the low glycemic index of the jicaro milk.

Mineral contents were similar to soybean, except iron which was much higher in jicaro seeds (159mg/kg vs 55mg/kg). However, compared to other oilseeds, mineral contents were much higher (Bernat et al., 2014).

In spite of all these advantages the jicaro seed is unexploited due to the three fundamental facts: the first is that it is a wild plant and there are no agronomic studies that encourage cultivation on an industrial scale, Secondly, is the lack of efficient technology to separate the seed from the fruit, since this process is made in most cases in an artisan way without the use of machinery, which limits its availability in the market. And thirdly the lack of technologies for its transformation.

The second part of this thesis tries to find some responses to these limitations. An innovative dehulling process that combines roasting and tempering is presented. The challenge in this work was to remove the seed coat to obtain the white cotyledons, which because of their brown color give a dark appearance to the obtained milk (as in the traditional beverage “horchata”). The light color is import in order to make a milk that is competitive with other vegetal milks. Additionally, removing the hull has shown to be an efficient way to increase the protein content and improve enzymatic digestibility in other seeds such as sesame (Johnson et al., 1979).

The results showed that the optimal roasting was achieved at 160 ºC for 150 s, with 90% of open seeds and lightness loss of less than 10% (i.e., $L \geq 72$). Preserving white color of cotyledons was a key condition for the milk process and was considered an optimization variable, along with the percentage of open seeds.

A kinetic model of dehydration and lightness was obtained and the relation between the moisture content and the number of open seeds was established. With this modeling, a set of constraints (more than 90% of open seeds, less than 10% of lightness loss) were set and from them the best values of the parameters were obtained.

Sudden dehydration allowed the opening of the seed coat when popping effect appeared. The ruptures appeared as marks over the surface of the seed coat. This effect has been observed in other products subjected to roasting, such as rice (Chandrasekhar and Chattopadhyay, 1988), peanut (Dean et al., 2014) or amaranth (Castro-Giráldez et al., 2012).

Another effect of roasting was that lipid bodies coalesced and formed a layer flowing around the inner surface of the cell wall, probably because the proteins (oleosins and caleosin) that stabilize the lipid bodies were denatured with the heat. As a consequence, lipid extractability was increased.

However, roasting had a negative consequence on the brittle structure of the seed. In this case, tempering was applied to harden the cotyledon and give elasticity to seed coat without affecting the color nor the aroma. The effect was shown in Section 4.3: when the moisture was too low the roasted jicaro seeds became a powder when passing through the mechanical dehuller and when it was too high the seed coats stuck to the cotyledons. The best results were obtained when conditioned at 10% moisture for 2 h.

Roasting has also a beneficial effect on the organoleptic properties of jicaro seeds and this is why it has been traditionally used in jicaro seeds. As a matter of fact, roasting has been used to improve flavor, aroma, color and palatability in many seeds and grains such as sesame, peanut, cashews, coffee (Agila and Barringer, 2012; Baggenstoss et al., 2008; Kato et al., 1981). An analysis of the jicaro volatile compounds before and after roasting was carried out to study its effect.

The unroasted jicaro seed has a pleasant sweet aroma, which was attributed to the ethyl-2-methylbutyrate. Even low concentrations contributed significantly to the scent of the jicaro. Ethyl-2-methylbutyrate imparts the apple notes, which is typical of some fruits (Pang et al., 2012; Zheng et al., 2012). Therefore, the jicaro aroma allows the manufacturing of products palatable for human consummation. In contrast, soybean has an undesirable flavor due to the presence of compounds such as iso-pentanol, n-hexanol, n-heptanol and 1-octen-3-ol which are responsible for green bean like odor,

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LE NUMERO 1 MONDIAL DU MÉMOIRES

this makes its use objectionable (Arai et al., 1967; Kato et al., 1981). This key compound increased during roasting with the temperature. Moreover, new aromas were formed as a result of roasting, such as pyrazines, a typical volatile compound appearing in this kind of treatment (Lykomitros et al., 2016). Pyrazines are produced due to the Maillard reaction from reducing sugars and amino groups (Jousse et al., 2002). Consequently, the concentration of these compounds decreased as a result of roasting.

The goal of this part of the thesis was to find an efficient way of extracting the cotyledon material from the jicaro whole seeds. The optimization of the process gave dehulling efficiencies of more than 75% (ratio of cotyledon isolated from the seed). This allowed the possibility of obtaining a jicaro milk with similar appearance (light color) than other vegetal milk, as opposed to the traditional beverage, which has a dark unattractive color.

A preliminary analysis was performed on the elaboration of the jicaro milk coming entirely from jicaro seeds cotyledons. This raw material does not require complex machinery or technologies for the manufacture of the milk. A good extractability of the protein was obtained in the aqueous medium, a result that is consistent with the type of proteins found in the physico-chemical analysis of the jicaro seeds. As a by-product of the subsequent filtering, an oil cake was obtained, also rich in proteins. This could be used in the production of cookies or snacks contributing to the added value of the proposed process.

The initial objective of maintaining all the good properties of jicaro seeds (nutritional value and pleasant aroma) along with a light color was attained: more than 6% fat content and more than 4% protein content with a color similar to that of the unroasted cotyledons.

Different soaking conditions were analyzed with no significant impact except for the fat content, which slightly decreased with the soaking time and the a-value: soaking water became greener as the temperature and time increased.

A clinical study on the glycemic index was performed as well to complement the results. Jicaro milk was demonstrated to have a low glycemic index, making it a good alternative in the diet of diabetic people.

There are still some points in the elaboration of the milk that require further study, namely, a more scalable filtering mechanism, the optimization of the homogenization

phase in order to obtain a more stable emulsion and the realization of a sensorial analysis of the milk to determine the acceptability of this product.

Chapter 6

Conclusions and Perspectives

This thesis contributes to the knowledge of the jicaro seed (*Crescentia Alata*) with the objective of valorizing it as a raw material for the developments of new products with high nutritional and sensorial quality.

The application of chromatographic, proteomic and physico-chemical analysis methods has revealed the high nutritional value of the jicaro seed, comparable to soybean, with the advantage of not having anti nutritional factors.

The composition, aroma and light color of the cotyledons were the elements considered in the study to propose a new methodology to obtain a vegetal milk from jicaro seeds. The main challenge was to find an efficient process for the removal of the seed coats.

The combination of roasting and tempering were the key operations for the achievement of the mentioned objective. The optimization of the roasting process achieved a seed opening ratio of 80% without any impact on the color of the cotyledons nor in the typical jicaro aroma, which was identified for the first time. With tempering, this was translated in a dehulling efficiency of 75%.

The final contribution of this thesis was to design a flowchart for the elaboration process of the jicaro milk, which not only provides the required nutritional and

organoleptic properties, but is also healthy as it is lactose-free and has a low glycemic index. This make it a competitive product when compared to other vegetal milks.

6.1 Perspectives

In order to extend the results of this work, several research perspectives deserve to be considered:

***Regarding the raw material**:*

A study of the fermentation process of the jicaro fruit, taking into consideration the harvest of the fruits, starting of fermentation, type of fermentation and biochemical changes.

Concerning the process of manufacturing vegetable milk:

Improve the physical stability of the emulsion either by:

- The use of stabilizers such as, xanthan gum, arabic gum or carboxymethylcellulose (CMC) in order to avoid creaming and sedimentation.
- The application of Ultra High Pressure Homogenization (UHPH). This technology besides proposing a more stable product ensures the sterility of the milk

Use enzymes in the processing of jicaro milk in order to enhance the extraction of oil and increasing protein solubility.

About diversification products are proposed some challenges

Add value to the developed product by enhancing the potential health benefits of its consumption:

- Jicaro milk fermentation and non-dairy yogurt with probiotics (i.e. *Lactobacillus reuteri* and *Streptococcus thermophilu*).
- Manufacture of cookies, snack or other products from the oil cake

Perform a sensory evaluation of the milk to evaluate its acceptance by the consumer.

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