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BIOCHEMICAL CHARACTERIZATION OF FLOUR FROM SEEDS OF *Camelina sativa* L. (Crantz) AFTER CHEMICAL EXTRACTION OF OIL

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Galasso I., Caruso I., Martinelli T., Pecchia P., Reggiani R., **Russo R.** and Mapelli S. Riscoperta la *Camelina sativa* una valida alternativa per il biodiesel. L'Informatore Agrario, supplement (2012) 16:38-40.

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ABBREVIATIONS

AA: Amino acid
ADF: acid detergent fibre
ADFIP: protein bound to ADF fraction
ADL: acid detergent lignin
Ala: alanine
ALA: α -linolenic acid
ANOVA: Analysis of Variance
CNR: Consiglio Nazionale delle Ricerche
CoA: co-enzyme A
CP: Crude protein
CS: *Camelina sativa*
DIM: 3,3'-diindolylmethane
DM: dry matter
EE: ether extract
EFSA: European and Food Safety Authority
EPA: eicosapentanoic acid
ESP: epithiospecifer protein;
FDA: Food and Drug Administration'
GA: gondoic acid
GHG: greenhouse gas
GRAS: generally recognized as safe
GSL: Glucosinolates
HPLC: High performance liquid chromatography
HPTLC: High performance thin layer chromatography
I3C: Indole-3-carbinol
IBBA: Istituto di Biologia e Biotecnologia Agraria
Ile: isoleucine
IP1: to inositol-1-phosphate
IP5: inositol penta-phosphate
IP6: inositol hexaphosphate
LA: linoleic acid
Met: methionine
NAD: nicotinamide adenine dinucleotide
NADP: (nicotinamide adenine dinucleotide phosphate)
NDF: neutral detergent fibre
NDFIP: protein bound to NDF fraction
NE₁: net energy of lactation
NRC: Nutrient Requirement of Dairy Cattle
OA: oleic acid
OPA: o-phataldialdehyde
Phe: phenylalanine

RUP: Ruminant Undegradable Protein

SBM: soybean meal

SDS-PAGE: sodium dodecyl sulphate- polyacrilamide gel

TCA: the citric acid

TLC: thin layer chromatography

Trp: tryptophan

Tyr: tyrosine

US: United States

Val: valine

ABSTRACT

Camelina sativa (CS) is an oilseed crop of the *Brassica* family that has gained increasing popularity as a biofuel source. The use of non-food plants as feedstock for biodiesel production is successful if the by-products, remaining after chemical extraction of oil, are valorized and utilized.

The study reported in this thesis was performed in order to characterize CS biodiesel by-product.

Some CS varieties different by origin were cultivated in two experimental fields: one set up in Casazza (BG) -Italy- in two years of cultivation and two growing seasons and the other in Firenze-Italy-. At the end of growing season, seeds were collected, dried, defatted and the flours used for biochemical analyses.

Meal protein content of nine genotypes from experimental field set up in Casazza (BG) was investigated to identify the genotype with highest protein content. The mean of meal protein content in two years of cultivation was 32.1% in defatted flour (d.f.) in autumn sowing and 36% d.f in spring sowing. ANOVA analysis showed that CS meal protein content was significantly different both for sowing season and year of cultivation. In spring sowing, seeds presented higher protein content respect to autumn sowing due to better nitrogen absorption in the warming season. Ligena was the genotype with highest protein content. SDS-PAGE of meal protein extract revealed the presence of two major bands: 27 and 15 kDa, likely belonging to cruciferin and napin fractions respectively.

Meal protein extract was subjected to acid hydrolysis in order to determine amino-acid composition of CS protein by HPLC (high performance liquid chromatography). The study of amino acids (AA) profile of CS protein revealed the presence of all AA, including the ten essentials. Among the essential AA, leucine and valine were predominant while lysine was the limiting determining the biological value of CS meal.

In order to evaluate the use of CS meal in animal feed diets, it was estimated the predictive digestibility of CS meal protein, in ruminants, by studies “*in vitro*” of ruminal protein degradation rates and RUP (ruminal undegraded protein) analysis. The digestibility of protein meal is strictly influenced by fibre content in seed coat in particular to the NDF (neutral detergent fibre, part of cell wall soluble in solutions at pH=7). For this, analyses of the fibre components of cell wall were performed. The significant differences of theoretical digestibility of protein among the CS genotypes, can be explained by fibre and NDIFP (protein bound to neutral detergent fibre fraction) content.

A limitation in the use of CS by-products in animal feed is the presence of anti-nutritive compounds (glucosinolates, sinapine, phytic acid and condensed tannins). From the analyses of twelve genotypes of CS from experimental field set up in Firenze, it resulted that sinapine, phytic acid and condensed tannins can be considered low in comparison with other members of Brassicaceae and not toxic for animal.

Glucosinolates (GSLs) represent the main anti-nutritive compounds in CS. Three main GSLs were indentified named GSL1 (9-methyl-sulfinyl-nonyl-GLS) glucoarabin, GSL2 (10-methyl-sulfinyl-decyl-GSL) glucocamelinin, and GSL3 (11-methyl-sulfinyl-undecyl-GSL) gluconesiliapanuculatin. GSL2 represented the most abundant GSL being between 50-60%. GSL content in CS ranged from 15.2 to 24.6 mmol/Kg (DM), with most

genotypes with less than 20 mmol/Kg DM. These values are higher respect those considered safe for animal. For this, it was necessary to develop a fast and reliable method to evaluate genotypic variation in GSL content to breed CS plants low in GSLs. A new HPTLC (high performance thin layer chromatography) procedure for screening CS genotypes allowed us to save time and solvent with respect to conventional HPLC procedure and thus represents a fast and economic alternative to HPLC for the determination of CS GSLs.

Therefore, CS meal presented high protein content, in particular during warming season, a balanced AA profile and a quite good ruminal digestibility of protein similar to rapeseed or soybean meal generally used in animal feed. Low-input new by-product biodiesel, CS, could strongly substitute high-input by-product biodiesel rapeseed or soybean as ingredient in animal feed formulations, as it occurred in U.S., where the introduction of CS meal in animal feed formulations was successful.

The characterization of CS meal described in this thesis suggests that an experimentation on the use of CS by-product in animal feed in Europe could be now undertake

1. Introduction

1.1 *Camelina sativa* (L.) Crantz, a new oilseed plant for biodiesel production

From several years, many national and international initiatives are underway in order to identify new sources of renewable energy. Renewable energetic sources attract a great interest, especially after guidelines of the European Parliament and EU council of March 23th 2009.

These guidelines provide three goals for 2020:

- Reducing energy consumption by 20%.
- Promoting renewable energy.
- Increasing the use of Biofuels, electricity, and hydrogen for transport with a minimum of 10% in every Member State.

This international growing interest in renewable energy is driven by two main reasons:

1. Depletion of fossil energy.
2. Emissions of dangerous greenhouse gases (GHGs).

Fossil fuels are: coal, crude oil and natural gas. Coal provides around 28% of our energy, Burning coal produces sulphur dioxide, an acid gas that contributes to the formation of acid rain. Crude oil (called "petroleum") is easier to get out of the ground than coal. Natural gas provides around 20% of the world's consumption of energy and, besides being burnt in power stations, is used by many people to heat their homes. They are not unlimited resources on our planet, the estimation time depletion for fossil energy will be around 50 years for the oil, 70 years for natural gas and 170 years for coal (International Energy Outlook 2006).

The combustion of fossil fuels emits large amounts of gas into the atmosphere, increasing the natural greenhouse effect. Carbon dioxide (CO₂), methane (CH₄), water vapor (H₂O) and ozone (O₃) are the components of greenhouse gases (GHGs). Between the period 1970 to 2004, GHG emissions (measured in CO₂-equivalent) increased at an average rate of 1.6% per year, with CO₂ emissions from the use of fossil fuels growing at a rate of 1.9% per year. Total anthropogenic emissions at the end of 2009 were estimated at 49.5 gigatonnes CO₂-equivalent. These emissions include CO₂ from fossil fuel use and from land use, as well as emissions of methane, nitrous oxide and other GHGs covered by the Kyoto Protocol. At present, the two primary sources of CO₂ emissions are from

burning coal used for electricity generation and petroleum used for motor transport (Reuters, 2009).

Electricity generation can derive from various renewable energy sources (wind, solar, geothermic) while the transport sector relies almost entirely on petrol. Therefore over the next 25 years, world demand for liquid fuels is expected to increase more rapidly in the transport sector than in other end-use sectors. The transportation sector has become the main target for renewable energies. Moreover, one of the guidelines of EU council on March 23th 2009 regards the use of biofuels in the transport sector encouraging the increase of consumption of biofuels in transport by replacing 10% of transport fuels in biofuels by 2020 (Directive 2009/28/EC).

In a context of growing interest for renewable energy, biodiesel defined as the mono-alkyl esters of vegetable oils, is an environmentally attractive alternative to conventional petroleum diesel fuel by the possible reduction of GHG emissions in the transportation sector. A recent report (International Grains Council 2008) indicated that rapeseed oil was the predominant feedstock for worldwide biodiesel production in 2007 (48%, 4.6 million metric tons, MMT). The remaining oils included soybean (22%, 2.1 MMT) and palm (11%, 1.0 MMT) with the rest (19%, 1.8 MMT) distributed among other unspecified vegetable oils (sunflower, palm oils, etc.). Feedstocks for biodiesel production vary with location according to climate and availability. Generally, the most abundant commodity oils in a particular region are the most common feedstocks. Thus, rapeseed and sunflower oils are principally used in Europe for biodiesel production, palm oil predominates in tropical countries, and soybean oil are most common in the USA (Demirbas, 2006). However, due to their value in the food sector, edible oilseed can be used only to a limited extent. To overcome this problem, several scientific studies are proposed in order to evaluate alternative oilseed plants that do not come in competition with food crops and present desirable characteristics for biodiesel production such as: high oil content, favourable fatty acid composition, low agricultural inputs (water, fertilizer, pesticides), adaptability to local growing conditions, growth in marginal soils, uniform seed maturation rate, potential market for agricultural by-products. One way to overcome the demand for oils and the need for alternative fuel oils is the use of non-edible oil seed plants.

The most interesting alternative oilseed plant are: *Jatropha curcas* (considerable interest as a feedstock for biodiesel production in India) *Pongamia pinnata*, *Brassica carinata*, and *Camelina sativa* (L.) Crantz (Moser, 2010). Among these non-food plants, *Camelina sativa*

well-adapted for temperate climates (North-America, Europe, etc.) and poor soils, is attracting great interest due to favourable agronomic and beneficial economic attributes: good yield/ha, growing in little fertile soils and not requiring agricultural practices (irrigation, fertilizers, herbicides and pesticides) and economically useful by-products.

1.2 *Camelina sativa* (L.) Crantz

1.2.1 Plant description

Camelina sativa (CS) (chromosome number $2n=40$, genome size 750 Mbp) (Hutcheon et al., 2010), is an ancient oilseed crop also known with different common names such as false flax and gold of pleasure (English) lendotter (German) and dorella (Italian). It belongs to the Cruciferae family (Brassicaceae). Some examples of Brassicaceae includes oilseeds like mustard, rapeseed, canola, crambe and vegetables like cabbage, cauliflower and broccoli. CS is a plant native from Northern Europe and Southeast Asia. According to archeological excavations, ssp of CS were known in the Bronze Age and in the Iron Age. During this period, the seeds of CS together with flax and cereals was a substantial part of the human diet. During the Middle Ages CS was grown sporadically. In the beginning of 20th century up to 1930s the crop was grown in France, Belgium, Balkan region and Russia (Zubr, 1997). Renewed focus on CS was inspired by the need of new vegetable sources of OMEGA-3 fatty acids or as an alternative oilseed plant for biofuel production. Recently, as feedstock for biofuel production, CS has attracted renewed interest due to some useful and distinguishable agronomic traits such as: very short growth cycle, resistance to drought and low temperature, capacity to growth in marginal soils and lower demand of fertilizers, herbicides and pesticides (Vollmann et al., 2007). It is currently extensively cultivated in Canada and U.S. In 2007, 10.000 hectares of the state of Montana were sown with CS (McVay and Lamb, 2008). Sporadic few hectares of cultivated CS have also been reported in the north and east Europe (Ireland, Germany, Austria, Poland and Slovenia). CS can be cultivated either over wintering or the spring growing cycle. On a taxonomic basis, CS is subdivided into three different subspecies *pilosa*, *sativa* and *foetida* (Angelini et al., 1998). From an agronomic point of view, CS ssp. *sativa* and ssp. *pilosa* appear to be the most promising subspecies. CS ssp. *pilosa* is usually characterised by vernalisation requirements to attain stem elongation and subsequent flowering. Given the susceptibility of this crop to cold stress during the stem elongation stage, ssp. *pilosa* appears to be more adaptable than *C. sativa* ssp. *sativa* to autumn sowing in temperate climates. CS is characterised by a high

level of morphological plasticity. In the field, plant size and the tendency towards branching are both influenced by general growing conditions and sowing density. The species is usually characterised by a short growing cycle (about 110 days in the spring cycle) that may be further shortened if adverse climatic conditions (i.e. drought) arise during plant development. CS seeds sown in the soil under favourable conditions (temperature, humidity) germinates within a few days, the emergence takes place within 7-10 days, with the appearance of two cotyledon leaves. Immediately after the cotyledonary stage, true leaves begin to develop, forming a rosette. The rosette later becomes a basis for an erect stalk with numerous leaves. The plants usually reach 70 to 100 cm in high. **(Figure 1.1)**

Figure 1.1: CS during flowering (Casazza field).



Such as most Brassicaceae, CS also develops lateral branches. In CS, the development of lateral branches is extremely variable and depends on the genotype, plant density and the environmental conditions. Therefore, the number of branches can vary from zero (with high plant density) to 27-30 (with low plant density and/or favorable climatic conditions) (Martinelli and Galasso, 2011). The inflorescence is a cluster of pale yellow flowers about 5-7 mm in diameter (**Figure 1.2a**). The flowers are mainly autogamous. The small pear shaped capsule with a tip (2-3 mm) contains about 15 oval shaped yellow seeds (**Figure 1.3b and 1.3c**). During and later under storage, the colour of the seeds turns dark brown.

The weight of 1000 seeds is 0.8-1.8 grams depending on variety, growth conditions, nutrition, etc. (Zubr, 1997).

Figure 1.2: (a) CS inflorescence showing open flowers. (b) Dry siliques before harvesting. (c) Open siliques with seeds



1.2.2 Seeding and nutrient requirements

Soil preparation is one of the most sensitive aspects of the CS growing technique. The preparation of a good seedbed is a necessary condition to obtain uniform germination. Therefore, after sowing at least one harrowing and one rolling are necessary in order to obtain a good seed-soil contact. Given the small size of the seed, the sowing depth should not exceed 2-3 cm with a row spacing of 13-15 cm. About 5-6 kg/ha of seed are required to obtain a population of 450-500 plants per m². Of all agricultural methods, balanced nutrition and fertilization of CS in particular predetermines the seed quality and oil yields. Nitrogen is one of the most important nutrients involved in the production of oilseed crops (Urbaniak et al., 2008). According to studies conducted in Europe (Zubr 1997) CS can be successfully grown with N levels of 100 kg/ha. Applications of nitrogen affect yield components, seed yield, oil and protein content (Urbaniak et al., 2008). Sulphur deficiency inhibits the plant use efficiency of N from fertilizers and may therefore increase N losses (Urbaniak et al., 2008). If the sulphur supply is insufficient, increasing rates in nitrogen will intensify this shortage and further reduce yields (Urbaniak et al., 2008).

1.2.3 CS yield

According to several reports, CS seed yield remains variable. Moser (2010) reported a seed production between 0.336 t/ha to 2.24 t/ha, while Vollmann et al., (2007) reported a seed production ranging from 1.57 t/ha to 2.25 t/ha. In Montana, where the cultivation of

CS is particularly extensive, yields of 1.7-2.4 t/ha were recorded (McVay and Lamb, 2008). The observed high variability in seed production is not only attributable to environmental and growing conditions, but also to the lack of selection and genetic improvement for this plant.

1.3 CS seed composition

1.3.1 CS oil

CS oil is the main product from CS seeds and the average yield of oil from the seeds is about 40% on DM basis (Zubr, 2009). It is a golden yellow colour liquid with a mild nutty and characteristics mustard aroma (**Figure 1.3**). Sensory test revealed a distinct flavour resembling fresh cauliflower. Some of the physical properties of CS oil reported are: refractive index 1.4756, density 0.92 g/cc both measured at 25°C, iodine number 105 (g I₂/100 g oil) and saponification value 187.8 (mg KOH/g oil) (Abramovic and Abram, 2005). Processing of the seeds on an industrial scale to release the oil, requires two steps, crushing and pressing. The temperature during pressing can reach up to 100°C. Prior to applications, the oil needs upgrading by deodorization. As a consequence of a natural purity of the oil, refining is very simple. Filtration is the only pre-treatment prior to deodorization (Zubr, 1992). The simple refining of the oil besides saving energy and expenses, avoids by-products such as polluted water and polluted bleaching soil.

Figure 1.3: CS oil.



CS oil is highly unsaturated. The oil contains about 64% polyunsaturated, 30% monounsaturated and 6% saturated fatty acids (FAs). Fatty acid (FA) content in CS oil depends mainly on the varieties and on the conditions under which the crop was grown (Zubr, 2009). The main FAs are: α -linolenic acid (18:3 n-3) (ALA), linoleic acid (18:2 n-6) (LA), oleic acid (18:1 n-9) (OA) and gondoic acid (20:1 n-9) (GA). The presence of GA is

a curiosity of CS oil. The role of this FA in human metabolism is not known. The content of erucic acid was 2.3-3.7% (Zubr, 2009). This was below the limit of 5.0% allowed in vegetable oils for human consumption (Zubr, 2009). The levels of erucic acid in human foods are partly restricted, since may adversely affect heart tissue. An early study had shown rapeseed oil, which contains erucic acid, promoted myocardial lesions in male adult rat animal models (Kramer et al.,1982). The ratio of LA (15%) and (ALA) (40%) is unique among the common vegetable oils such as soya oil, sunflower oil, rape oil, olive oil etc.

The oil also contains high levels of gamma-tocopherol (Vitamin E) which confers a reasonable shelf life without the need for special storage conditions. The total content of tocopherols in CS oil ranged 800-900 µg/g. This was higher than flax oil and rape oil.

1.3.2 Exploitation of CS oil

From the nutritional point of view, CS oil is a rich source of essential fatty acids (LA and ALA). Animal research suggests that CS oil may have a significant effect on the reduction of triglycerides and cholesterol in pig serum. Eidhin et al., (2003) concluded that the CS oil diet increased ω -3 long chain FAs, in particular eicosapentanoic acid (EPA) and improved the ratio ω -6/ ω -3 FAs in plasma. Potential health benefits of omega-3 from CS oil are being evaluated in a breast cancer risk study for overweight or obese postmenopausal women. Because of its nutraceutical effects, the oil could attract considerable attention for use in the production of health promoting foods (Abramovic and Abram, 2005). The oil was found to be applicable in salads, for cooking, baking and frying, except for deep-frying. Applicability of the oil in certain food products, such as mixed fats, mayonnaise, dressings, icecream, etc., was confirmed by experiments on a laboratory and pilot scale. Recently, Raisio Nutrition Ltd has supplemented the food products with CS oil starting in year 2003 with margarine products (Aronen et al., 2003).

Due to its outstanding purity, CS oil was earlier used for lighting. Industrially, the oil was exploited for production of soaps and varnishes. The specific dermatological effects of polyunsaturated FAs make CS oil suitable for cosmetic applications, such as cosmetic oils, skin creams and lotions (Hurtaud and Peyraud, 2007). Moreover, CS oil can be converted to a wax ester that will replace more expensive and less available Jojoba waxes in arrange of industrial and cosmetic products (Mc Vay and Lamb, 2008).

Non-food exploitation of CS oil was found in biofuel. In spite of a high iodine number (about 105), successful experiments on a pilot scale were carried out with conversion of the oil into methyl-ester. The results indicate that the methyl-esters produced from CS oil has properties similar to rape methyl-esters. Fuel consumption and general vehicle operation with CS ester are similar to that reported for rape methyl ester (Frohlic and Rice, 2005). In Montana, CS biodiesel has been produced and evaluated by commercial biodiesel manufactures including Core IV, Wyoming Biodiesel Peaks and Prairies and Great Northern Growers. CS biodiesel performance appears to be equal in value and indistinguishable from biodiesel produced from other oilseed crops such as soybean (Mc Vay and Lamb, 2008). As feedstock for jet bio fuel, CS meets all the key properties of fossil derived aviation fuel. Under ASTM D4054 (issued 1st September 2009) and certification D7566 (December 2010) CS jet fuel has been approved as biodiesel and jet kerosene thus making jet fuel derived from CS and Jatropha the choice of the US Navy, Air Force along with 12 U.S Airlines (IATA, 2011) In 2009, both Japan Airlines (JAL) and KLM Royal Dutch Airlines successfully flight-tested a 50% blend of hydrotreated renewable jet fuel (HRJ) derived from the cultivation of CS (Shonnard et al., 2010).

1.3.3 CS meal

CS meal is the product obtained from high-pressure crushing of seed or from a pre-press solvent extraction process, which removes the oil from the whole seed (**Figure 1.4**). It represents an important output with considerable economic value.

Figure1.4: CS meal.



CS meal consists of 13% residual oil, 6% ash, 12% crude fibre, 30% crude protein, 27% non-nitrogenous matter and other substances such as vitamins etc. The meal has a metabolizable energy as high as 1600 per pound (Zubr, 1997).

1.3.3.1 Seed protein

In CS meal, protein content is about 30-35% DM basis. A large part of this percentage are seed storage proteins. There are few data in literature about CS seed storage proteins. In Brassicaceae oilseeds, two classes of seed storage proteins predominate: legumin-type globulins (12 S or cruciferin) and napin-type albumins (2S or napin). They constitute 60 and 20% respectively, of the total proteins in mature seeds (Wanasundara et al., 2010). The 12S proteins of Brassicaceae oilseeds have Mr of 30-36 kDa and is composed of six subunits (hexamer) that are arranged as two trimers to be hold together by salt bridges. Each subunit of this hexameric assembly is composed of acidic and basic polypeptides linked with one disulfide bond (Adachi et al., 2003). The 2S protein of Brassicaceae oilseeds has Mr of 15-18 kDa and is composed of a heavy/large (10-12 kDa) and a light/small (4-6 kDa) polypeptide that are linked by four disulfide bonds (two inter- and two intra chain) (Rask et al., 1998). The 2S albumins or napins in oilseed rape and turnip rape are potential food allergens (Puumalainen et al., 2006). The 12S and 2S proteins are different in molecular structure, amino acid composition, and physico-chemical properties and are therefore able to provide different functionalities in practical applications.

1.3.3.2 Carbohydrates

Carbohydrates of CS include monosaccharides, disaccharides, oligosaccharides, polysaccharides and fibre. Monosaccharides and disaccharides are easily digestible and in the human body provide easily metabolisable energy. The content in CS is very small, for example sucrose is about 5.5%, it was twice as high as flaxseed (2.8%) but lower than rapeseed (6.8%) (Knudsen and Betty, 1991). Oligosaccharides: raffinose and stachyose are very low in CS (below 1%) (Zubr, 2010). They are poorly digested in the small intestine. But they are fermented in large intestine by microflora producing gas. Polysaccharides: starch, pectin and mucilage. Starch is a polysaccharide consisting of different chain length and straight chained amylose and branch chained amylopectin. The content in CS is very low (1%) (Zubr, 2010). Starch is incompletely digestible in the small intestine, but it is fermented by microbes in the large intestine. Pectin is a heteropolysaccharide consisting mainly by d-galacturonic acid linked with fucose, xylose and galactose. This fermentable fibre is very low in CS less than 1% (Kitts, 2007). Mucilage is a water soluble fibre that forms gel. Soluble fibres delay gastric emptying and transit through the colon. Soluble fibres interfere with the absorption of sugars and fats. They absorb potentially noxious

carcinogenic compounds of the ingesta (Berdanier, 2000). The content of mucilage in CS is 6.7%, lower than flaxseed (8%) (Zubr, 2010). Crude fibre, include cellulose and hemicelluloses. Cellulose is a non-digestible glucose polymer. It is found in the cell wall of all vegetation. Hemicellulose fibres are cellulose molecules substituted with other sugars such as xylan galactan, mannan, etc. Cellulose and hemicelluloses are microbially fermented in large intestine. A mixture of short chain fatty acids, such as acetate, butyrate and propionate are produced (Kitts, 2007). Lignin is a polyphenolic compound associated with dietary fibre. It is water insoluble and in the gastro intestinal system, it increases the amount of stool and absorption of water (Slavin, 2007). The content of lignin in CS is 7.4% (Zubr 2010). The content of crude fibre in CS meal is about 15% DM basis. The substantial part of crude fibre was cellulose. The proportionally high content of mucilage, crude fibre and lignin indicates that CS meal, when incorporated in food, can exert positive effects on gastrointestinal processes. A long term human consumption of bread with added CS meal confirmed that beneficial role of the ingredient in digestion (Zubr 2010).

1.3.3.3 Vitamins and Minerals

CS meal is a good source of vitamins B₁ (thiamin), B₃ (niacin) and B₅ (pantothenic acid). Thiamin in nature exists as thiamine pyrophosphate. It functions as a coenzyme in transketolation and is important in neural transmission. It is directly involved in maintenance of normal appetite and healthy attitude (Berdanier, 2002). The content of thiamin in CS is considerable higher (18 µg/g) respect than flaxseed (6 µg/g) and rapeseed (8 µg/g) (Zubr, 2010). Niacin occurs in two forms as nicotinic acid and nicotinamide. It is widely distributed in nature but it does not occur in large amount in free form. Most often it is found as the coenzyme NAD⁺ (nicotinamide adenine dinucleotide) and NADP⁺ (nicotinamide adenine dinucleotide phosphate). Niacin is one of the most important vitamins in human and animal nutrition (Zapsalis and Beck, 1985). The content of niacin in CS (194 µg/g) is predominant among the vitamins, it results also about twice as high as in flaxseed (91 µg/g) (Zubr, 2010). Panthotenic acid has diverse metabolic functions as a structural component of coenzyme A (CoA) and acyl carrier protein. The CoA supports the transmission of nerve impulses, haemoglobin synthesis, synthesis of sterols and steroid hormones, maintenance of normal blood sugar, formation of antibodies etc. (Berdanier, 2002). The content of panthotenic acid is identical to flaxseed (11 µg/g) and lower than rapeseed (16 µg/g) (Zubr, 2010).

Zubr (2010) reports that CS meal is a marginal source of mineral except the micro-minerals iron, manganese and zinc. Analyses of CS reveal a prevalently low content of macro-minerals. The highest content between 1.0-1.6 % is calcium, potassium, phosphorus. (Zubr, 2010). Among micro-minerals, CS presents markedly high content of iron (329 µg/g), manganese (40 µg/g) and zinc (69 µg/g) (Zubr 2010).

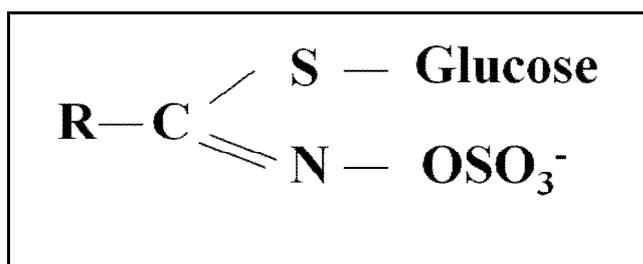
1.3.4 Anti-nutritive compounds

CS meal is characterized by the presence of minor substances that affect the value of this by-product. Especially plant secondary metabolites such as glucosinolates (GSLs), sinapine, inositol phosphates and condensed tannins belong to widespread anti-nutritive compounds which are generally present in oilseeds. GSLs and sinapine have usually been associated with members of Brassicaceae whereas inositol phosphates and condensed tannins are more generally distributed in flora (Russo and Reggiani 2012a).

1.3.4.1 Glucosinolates (GSLs)

The most important anti-nutritional compounds found in Brassicaceae seeds like CS are GSLs. GSLs are β-thioglucoside N-hydroxysulphates with a side chain (R) and sulphur linked β-D-glucopyranose (**Figure 1.5**).

Figure 1.5: GSL structure



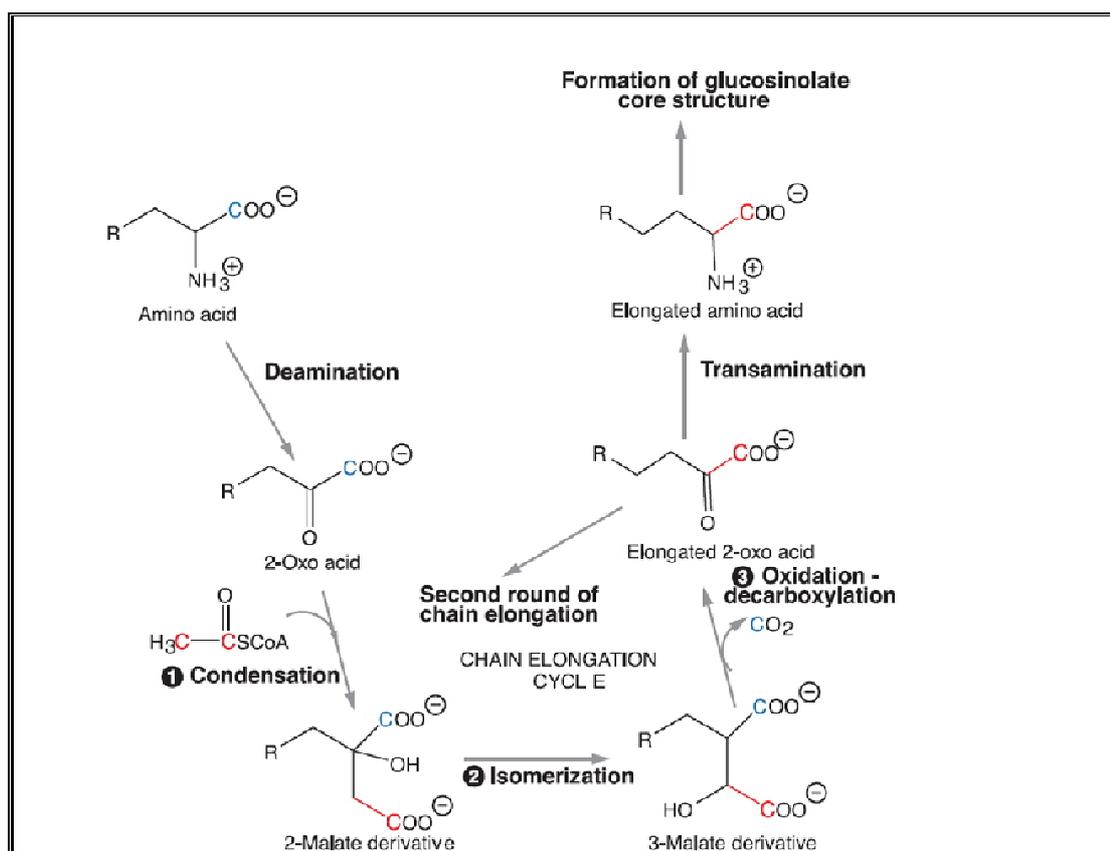
R= side chain

They are hydrophilic and rather stable molecules and remain in the press-cake of oilseeds when this is processed and defatted. Oil extraction process affects the total GSL content of the meal because of varying oil extraction conditions. Solvent-extracted meals contain higher amount of GSL than that in dehulled extracted meals. The GSL structure was originally confirmed by X-ray investigations on synthesized sinigrin (Marsh and Waser, 1970). This structure is found to be a general feature of all GSLs investigated by ¹H

and ^{13}C -NMR spectroscopy (Bellostas et al., 2007). GSLs can be classified by their precursor and the type of modifications of R group. Compounds derived from alanine (Ala), isoleucine (Ile), methionine (Met) or valine (Val) are called aliphatic-GSLs, those derived from phenylalanine (Phe) or tyrosine (Tyr) are called aromatic-GSLs and those derived from tryptophan (Trp) are called indole-GSLs. The R groups of most GSLs are extensively modified from these precursor amino acid. Most of the R groups are elongated by one or more methylene moieties. Both elongated and non-elongated R groups are subject to a wide variety of transformations including hydroxylation, methylation, desaturation, glycosylation and acylation.

Biosynthesis of GSLs is illustrated in **Figure 1.6**. The sequence of the chain-elongation pathway for amino acids participating in GSL biosynthesis is based on in vivo feeding studies, the demonstration of enzyme activities in vitro, and the isolation of key intermediates. Initially, the parent amino acid is deaminated to form the corresponding 2-oxo acid. Next is a three-step cycle in which (1) the 2-oxo acid condenses with acetyl-CoA to form a substituted 2-malate derivative, which then (2) isomerizes via a 1,2-hydroxyl shift to a 3-malate derivative that (3) undergoes oxidation-decarboxylation to yield a 2-oxo acid with one more methylene group than the starting compound. During each round of the elongation cycle, the two carbons of acetyl-CoA are added to the 2-oxo-acid and the COOH group added in the previous round is lost, for a net gain of one carbon atom. After each turn of the cycle, the extended 2-oxo acid can be transaminated to form the corresponding amino acid and enter the second phase of GSL formation. Alternatively it can undergo additional cycles of acetyl-CoA condensation, isomerization, and oxidation-carboxylation, resulting in further elongation. Up to nine cycles are known to occur in plants. Similar 2-oxo acid-based chain-elongation sequences occur in leucine biosynthesis and in the TCA (the citric acid) cycle, as well as elsewhere in plant metabolism (Halkier and Gershenzon, 2006).

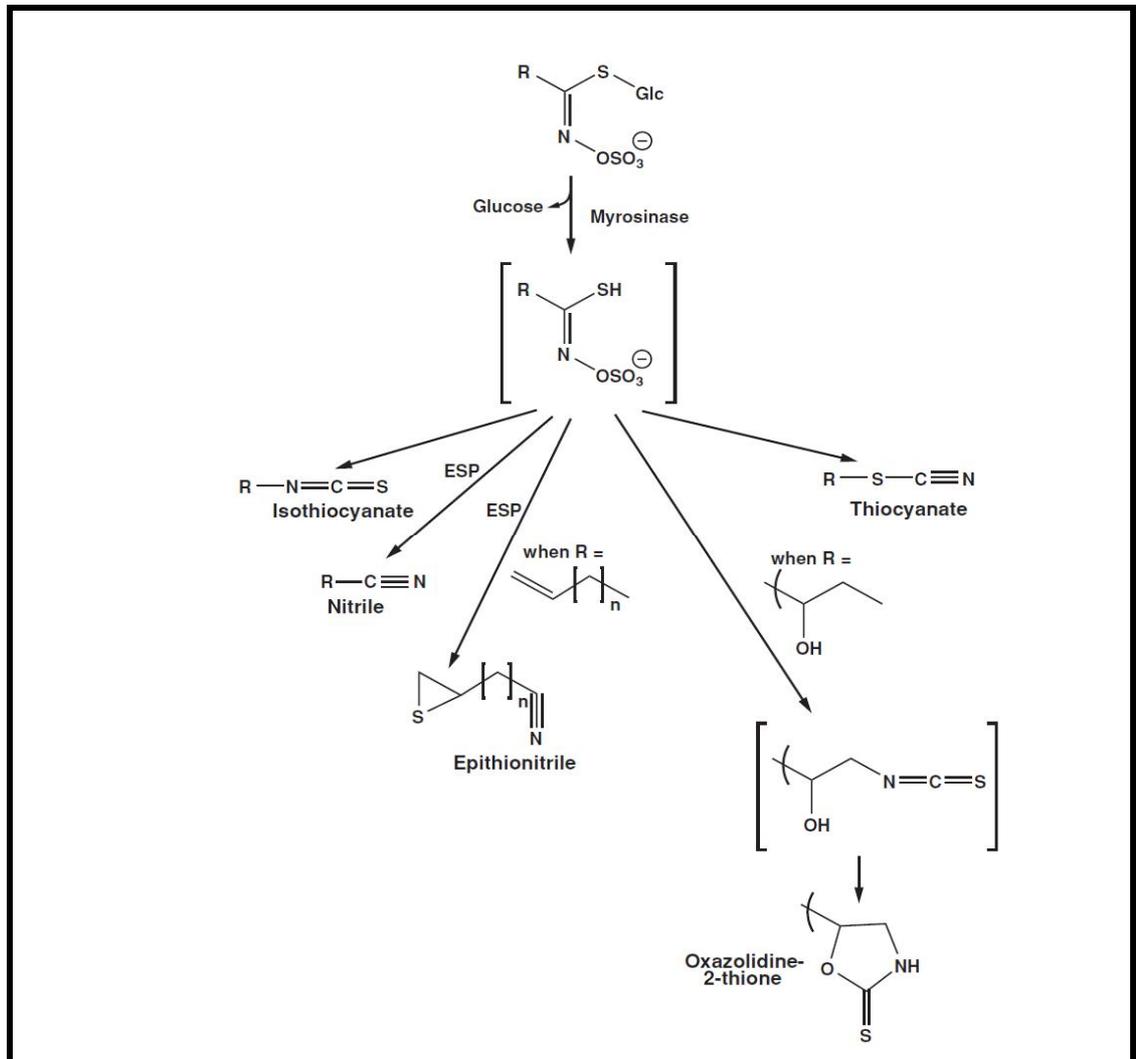
Figure 1.6: Amino acid chain elongation cycle for GSL biosynthesis. Illustrated is the first round of elongation. The three principal steps are: (1) condensation with acetyl-CoA, (2) isomerisation, and (3) oxidation-decarboxylation. The carbon atoms contributed by acetyl-CoA (retained with each round) are shown in red. The carbon atom from the original COOH function (lost with each round) is shown in blue.



GSLs present in all plant parts, are part of innate defence system. They convey different signals to herbivorous insects in attracting parasitic wasps and favouring or opposing oviposition by insects. The defence systems involves thioglucosidases commonly known as myrosinases. In the intact plant, GSLs and myrosinases are sequestered in different compartments. Matilde (1980) concluded that the stability of GSLs in plants appeared to be due to the location of GSLs and myrosinase in distinct subcellular compartments of the same cell. These cells are named myrosin cells. Myrosinase in extracellular compartments (cell walls) and associated with the cytoplasmic side of internal membranes, while GSLs were localized in vacuoles. Upon plant damage, the enzymatic reaction takes place (GSLs become accessible to myrosinases) resulting in the formation of breakdown products: isothiocyanates, thiocyanates, oxazolidine-2-thione, epithionitriles and nitriles (**Figure**

1.7). GSL are hydrolyzed by both myrosinase enzyme present in plant and myrosinase produced by intestinal microflora.

Figure 1.7: Outline of GSL hydrolysis. Brackets indicate unstable intermediates. Abbreviations: ESP, epithiospecifer protein; R variable side chain.



1.3.4.1.1 Biological effects of dietary GSLs

a) Toxicity of GSLs

GSLs themselves are biologically inactive molecules, but GSL degradation products are biologically active and known for their diversified biological effects. The toxicity of GSLs is generally attributed to the isothiocyanates, thiocyanates, oxazolidinethiones and nitriles originating from enzymatic cleavage of GSLs by myrosinase. Thiocyanate and oxazolidinethione anions are known to compete with iodine in two ways, by inhibiting its

uptake through competition with the sodium-iodide symporter, and binding of iodine to tyrosine residues of thyroglobulin at high concentrations (De Groef et al., 2006). Hence one of the common symptoms of GSL exposure is the impairment of the thyroid function, resulting in a hypertrophy of this endocrine gland (goitre). Clinical signs for hypothyroidism following exposure to toxic concentrations include, reduced feed conversion and impairment of growth, fertility and reproduction (Conaway et al., 2002; EFSA, 2008). In addition, irritation of the gastrointestinal mucosa followed by local necroses, hepatotoxicity and nephrotoxicity have been observed, commonly attributed to the presence of nitriles (EFSA, 2008).

b) Beneficial effects of GSLs

Like most other vegetables, cruciferous vegetables are good sources of variety of nutrients and phytochemical that may work synergically to help prevent cancer. An extensive review of epidemiologic studies published to prior 1996 reported that the majority (67%) of 87 case-control studies found an inverse association between some type of cruciferous vegetable intake and cancer risk. At that time, the inverse association appeared to be most consistent for cancers of the lung and digestive tract (Hossain and Rahaman 2011). Among the GSLs breakdown products, two appear to have anti-tumoral properties: isothiocyanates and indole-3-carbinol. Isothiocyanates may help to prevent cancer by enhancing the elimination of potential carcinogens from the body increasing the transcription of tumor suppressor proteins, including those silenced by epigenetic mechanisms. Indole-3-carbinol (I3C) in the acidic environment of the stomach forms a number of biologically active indole acid condensation products, such as 3,3'-diindolylmethane (DIM) and related oligomers. I3C has been found to inhibit the development of cancer in animals when given before or at the same time with carcinogen (Stoner et al., 2002).

c) Adverse effects of GSLs in animal nutrition

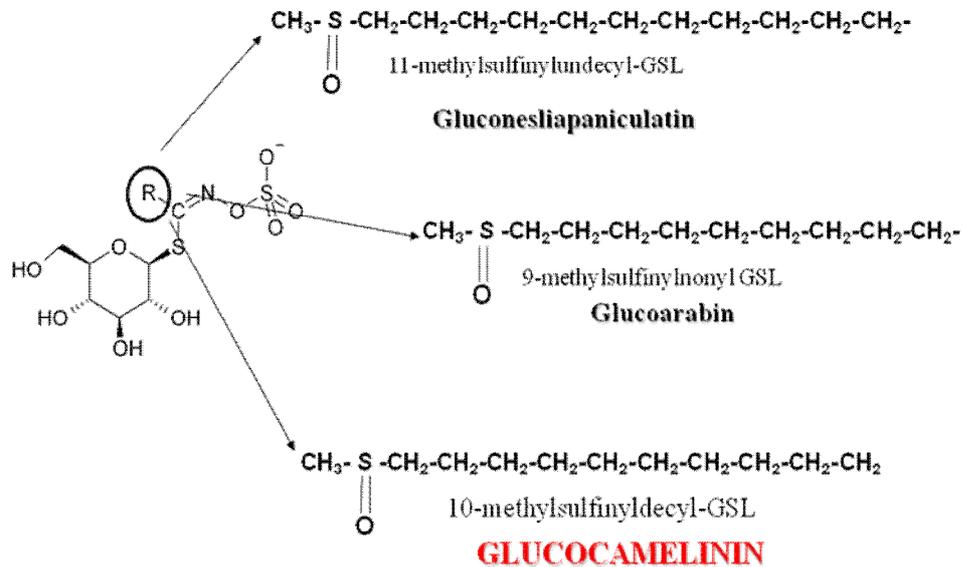
Intoxication following the consumption of GSL-containing plants have been described in all major farm animal species. The degree of adverse effect of dietary GSLs depends on the level and composition of GSLs and their breakdown products. Different animal species have varying GSLs tolerance capabilities (Tripathi and Mishra, 2007). Pigs are among the most sensitive animal species regarding acute adverse effects of GSLs. Typical effects attributable to rapeseed meal were a delayed sexual maturity and decrease in the number of

piglets born alive (Schone et al., 1997a). Previous experiments have indicated that diets with a total GSLs level below 1 mmol/kg did not induce significant adverse effects, whereas levels exceeding 1.34 mmol/kg resulted in reduced feed intake and growth. Dietary levels between 9-10 mmol/kg induce iodine deficiency and an increase in the serum level of T₃ and T₄ followed by thyroid hypertrophy (Mawson et al., 1994b). Deleterious effects of dietary GSLs were observed on growth and performance among commercial domestic birds. The high GSL level diet also significantly reduced egg production and plasma urate levels. At lower concentrations, GSLs decreased only feed intake and weight gain (EFSA, 2008). Ruminants are considered to be less sensitive to GSLs in their feed as compared to monogastric species, since the rumen flora degrades various GSLs breakdown products (EFSA, 2008). GSL poisoning in cattle has been reported (Katamoto et al., 2001). Clinical symptoms include poor productivity, reduced fertility and poor body condition. Cows showed signs of toxicity and thyroid dysfunction and depressed fertility following a daily intake of 44 mmol/day (Ahlin et al., 1994). A report of GSL poisoning in lambs mentions swayback, anaemia and visibility enlarged thyroid glands as major clinical signs. Lambs feeding on Brassica developed hypothyroidism. Body weight losses were reported in sheep following a GSL intake of 2.5-7.6 mmol/day (EFSA, 2008). Ewes showed an impaired fertility with significantly reduced oestradiol levels following dietary exposure to 1.2 -1.6 mmol/kg dry matter (Mandiki et al., 2002).

1.3.4.1.2 GSLs in CS.

In CS the total content of GSLs has been reported in some studies. Matthaus and Zubr (2000) reported a range between 14.4-23.4 mmol/kg while for Schuster and Friedt (1998) the total GSL content varied between 13.2-36.2 mmol/kg. Russo and Reggiani (2012a) reported a total glucosinolate content that varied between 15.2-24.6 mmol/kg dry matter (DM), with most genotypes with less than 20 mmol/kg. This content is higher (up 2 times) than the concentration in double-null rapeseeds, but much lower than other Cruciferous oilseed like Crambe (100 mmoles/Kg) and Mustard (120 mmoles/Kg) (EFSA, 2008). In contrast to other *Brassica* crops, the major seed CS GSLs have long aliphatic side chain. Three main GSLs were indentified named GSL1 (9-methyl-sulfinyl-nonyl-GLS) glucoarabin, GSL2 (10-methyl-sulfinyl-decyl-GSL) glucocamelinin, and GSL3 (11-methyl-sulfinyl-undecyl-GSL) gluconesiliapanuculatin (**Figure 1.8**). GSL2 represented the most abundant GSL being between 50-60% (Russo and Reggiani 2012a).

Figure 1.8: GSLs in CS. Abbreviations: R, side chain.

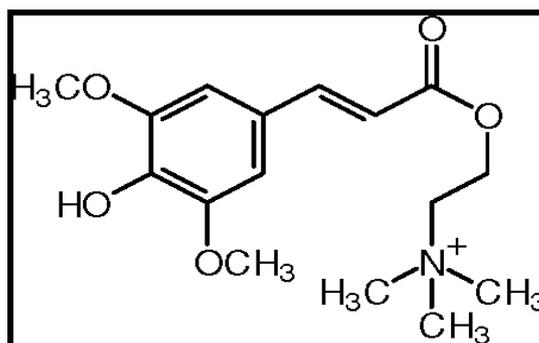


GSLs content and profile in *Brassica* species are influenced either from plant factors (such as species and developmental stage) and environmental conditions (Cartea et al., 2008). The latter include nutrient availability, especially N and S supply (Yan and Chen, 2007). Sulphur has a strong influence on GSL content in *Brassica* species and an increase in S supply resulted in all cases in a significant increase of GSL content. Sulphur has a direct role in GSL biosynthesis, because sulphur containing amino-acids are required as starter molecules for their synthesis. This is more important for aliphatic than indolyl GSLs because three atoms of sulphur instead of two are needed for their biosynthesis. On the other hand, data on the effect of N supply to the GSL content in plants appear rather contradictory. For example increase of N supply resulted in an increase of indolyl GSL content in *Brassica rapa*, (Yan and Chen, 2007) whereas Li et al., (2006) observed a decrease of aliphatic GSL content in fresh turnip roots by increasing nitrogen supply dose. Falk et al., (2007) found that aliphatic GSL remained constant by increasing N supply, although clear effects were described in all these studies. It is obvious that exists species-specific responses to the N and S supply and the environmental parameters may influence these responses like, for example, the growth period and plant tolerance to salts.

1.3.4.2 Sinapine (3,5-dimethoxy-4-hydroxycinnamoylcholine)

Sinapine is an alkaloidal amine (**Figure 1.9**) isolated for the first time in 1825 from black mustard seeds. It is a choline ester of sinapic acid widely distributed among the members of the Cruciferae family.

Figure 1.9: Sinapine



Sinapine accumulates as a reserve material during seed development. It is hydrolyzed during germination by aromatic choline esterase into choline and sinapic acid. Both hydrolysis products of sinapine appear to have metabolic importance in higher plants, sinapic acid for biosynthesis of lignin and flavonoids and choline for its function in the methylation cycle for membrane synthesis (Clausen et al., 1985). Sinapic acid is the predominant phenolic acid in rapeseed and it has been reported to be the most antioxidative component of canola meal (Wanasundara et al., 1994). Recently, the use of sinapine as an anticarcinogen has been suggested due to its very strong radiation protection effects (Li and El Rassi, 2002). The capacity of a plant to accumulate sinapine in the seed is correlated with seed size: bigger seeds accumulate more sinapine (Wang et al., 1998). Moreover, sinapine content per seed of stressed plants was 30-40% lower than that of well-watered plants, and is higher in plants treated in darkness compared with the same growing under normal light conditions (Kvartshava et al., 1980). The effects of the other environmental factors such as temperature, soil fertility and disease on sinapine content per seed have not been reported.

Sinapine and other related phenolic choline esters are responsible for the disagreeable taste of eggs produced by poultry fed with rapeseed meal. This occurs because during digestion, sinapine is hydrolyzed and the resulting choline is degraded to give trimethylamine (TMA) from microorganisms. Brown-egg laying hens devoid of liver trimethylamine oxidase do not detoxify this amine which is stored in the eggs giving the

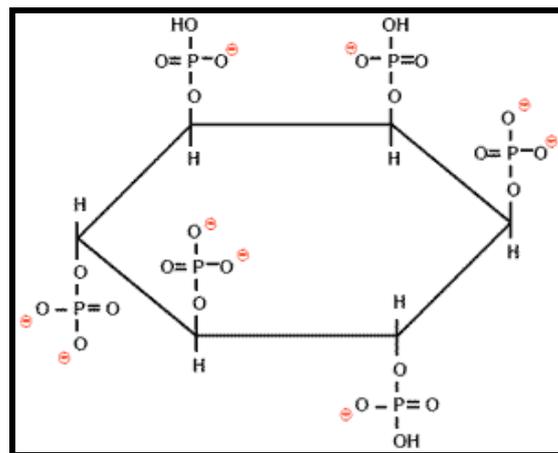
characteristic unpleasant smell (Andersen and Sorensen, 1985). These undesirable flavors and taints are attributed to sinapine which constitutes 1 to 4% of oil-free meal (Uppstrom and Johansson, 1985).

The amount of sinapine in CS seed was very low, about 2.3-3.0 mg/g DM (Matthaus, 1997, Russo and Reggiani, 2012a). It is significantly lower when compared with other Brassicaceae, such as rapeseed or mustard (7 and 13 mg/g respectively) (Matthaus, 1997). Thus, some adverse effects of sinapine from CS meal appears unlike.

1.3.4.3 Inositol Phosphates (Phytic acid)

Phytins, the mixed calcium (Ca) and magnesium (Mg) salts of myo-inositol 1,2,3,4,5,6-hexakis-dihydrogen-phosphate, also known as phytic acid are widespread in nature (**Figure 1.10**).

Figure 1.10: Phytic acid



They are the principal form of phosphorus (P) in many seeds; 60-90% of all the P in seeds is present as phytic acid. Several physiological roles have been suggested for phytic acid in plants. (Duff et al 2006) It may be used as P store, as energy store or as an initiator of dormancy. Duff et al., 2006 reported evidence that phytic acid serves only as a source of P and cations for the germinating seed. Urbano et al., 2000 found that phytate contains over 80% of the total P of mature rice grain and the turnover of phytate P is practically nil in the resting grain. From that, they concluded that phytate can be considered a final product of P metabolism in the ripening process accompanied by other storage substances such as lipids and starch. The accumulation sites of phytic acid in dicotyledonous seeds are in the globoids (which are inclusions of the protein bodies). The proportion of phytic acid reaches up to 60-80% of the dry weight of globoid in dicotyledons (Urbano et al., 2000).

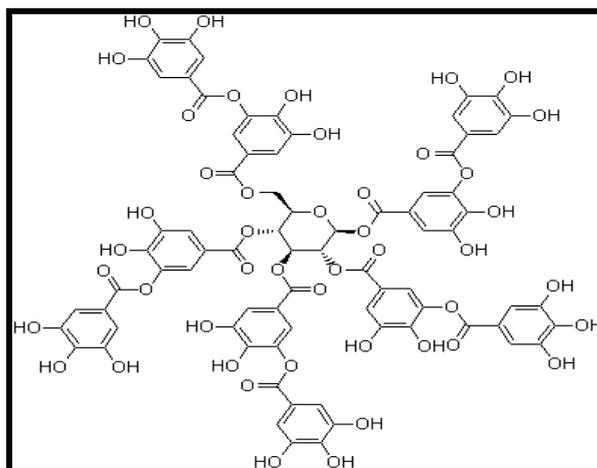
In animal, and in human nutrition, inositol hexaphosphate (IP6) is responsible for different anti-nutritive effects such as forming insoluble complexes with nutritionally important minerals (Fe, Zn, Mg, Ca). The mechanism by which phytate affects mineral nutrition is not clearly understood. It was only suggested that the formation of insoluble phytate-metal complexes in the intestinal tract prevents metal absorption. This is due to the strong chelating properties of phytic acid, interaction with proteins or the formation of complexes with digestive enzymes (Urbano et al 2000). In the formation of protein- phytic acid complexes, several studies indicate involvement of some side chains of proteins (Hidvegi and Lasztity, 2003). At a low pH, below the isoelectric point of proteins, the terminal amino, lysyl, histidyl and arginyl groups are positively charged. Any of these groups can directly form a complex with a negatively charged phytate anion. If the steric conditions are satisfactory, one phytate anion can interact with two charged groups of protein. Naturally, the protein molecule can bind more phytate anions at the same time, depending on the number of positively charged groups and conformational conditions. At intermediate pH values, only the lysyl and arginyl groups are positively charged, so in this case a slight possibility of electrostatic interactions exists. If the pH is very high, the interaction between phytic acid and protein is low. During food processing, storage and germination of seeds, IP6 is chemically or enzymatically dephosphorylated. Degradation products with less phosphate bound to the inositol ring are formed inositol penta-phosphate inositol pentaphosphate (IP5) to inositol-1-phosphate (IP1). The ability of these products to form complexes with minerals or proteins is much lower than that of IP6 (Mattahus and Zubr, 2000). Additionally to the anti-nutritive effects of inositol phosphates, it was shown in recent research that IP6 prevents and possibly reverses carcinogenesis, that is considered to work as an hypocholesterolemic agent by preventing renal stone formation as well as an antioxidant compound (Jariwalla et al., 1990).

In CS the content of phytic acid has been reported. For Mathaus and Zubr (2000) ranged between 21.9-30 mg/g DM, while for Russo and Reggiani (2012a) 25.4-32.3 mg/g DM.. The variation in the content of inositol phosphates can be associated to cultivar, origin of sample, climatic conditions and year (Hidvegi and Lasztity, 2003).

1.3.4.4 Tannins

Tannins are water-soluble phenolic compounds showing molecular weight between 500 and 3000 Da. (**Figure 1.11**)

Figure 1.11: Tannins, general feature.



Tannins are present in a variety of plants utilized as food and feed (grains, sorghum, faba beans, pigeon peas, etc.) a fruits (apples, bananas, blackberries, pears, etc.) and forages (crown and vetch) They are divided into three main classes: The condensed tannins or proanthocyanidine (flavan-3-ol based biopolymers), gallotannins and ellagitannins. Gallotannins are comprised of galloyl esters of glucose or quinic acid whereas ellagitannins are derivates of hexahydroxydiphenic acid (Hagerman et al., 2005). Condensed tannins are the polymerized products of flavan-3-ols and flavan-3,4-diols, or a mixture of the two. The polymers, referred as flavolans, are popularly called condensed tannins (more recently called proanthocyanidins). The flavan-3-ols are often referred to as catechins. Since catechin molecules possess two asymmetrical carbon atoms at the C-2 and C-3 positions, four isomers exists. There are (+) and (-) catechins, in which 2-phenyl and 3-hydroxy groups are trans. Flavan-3,4-diols belong to a class of compounds called leucoanthocyanins. A flavan-3,4-diol molecule possesses asymmetric carbon atoms at C-2, C-3, and C-4, hence eight isomers are present (Chung et al., 1998). Tannins are found in nearly every part of the plant, such as in the bark, wood, leaves, fruit, roots and seed. Frequently, an increased tannin production can be associated with some sickness of the plant. Therefore, it is assumed that the biological role in plants of many tannins is related to protection against infection, insects or animal herbivore (Khanbabaee and vanRee, 2001). The key feature that gives to tannins their characteristics properties seems to be an unusually high local concentrations of *ortho*-phenolic hydroxyl groups. In hydrolysable tannins, these are associated mainly with gallic or ellagic acid residues. In condensed tannins, ring B phenolic hydroxyls plays this role. Thus, the biological activity of tannins may be dictated, at least in part, by the molar content of these *ortho*-phenolic hydroxyl groups (Schofield et al., 2001).

Phytochemists and Nutritionists are used to call tannins “a double-edged sword in biology and health”. Tannins act as an anti-nutritive compound of plant origin because they form complexes with proteins, starch and digestive enzymes and reduce the nutritional values of foods. Animals fed with a tannin-free diets had higher feed consumption and weight gains compared with those fed with a diet containing endogenous or supplemented tannins for an extended period of time. In laying hens, long-term consumption of tannin-rich faba bean resulted in a decrease in efficiency of feed utilization and increased mortality (Chung et al., 1998). Tannins, particularly the condensed type, are reported to inhibit virtually every digestive enzyme including pectinase, amylase, lipases, proteolytic enzymes, β -galactosidase, cellulase. However, it is generally believed that the major dietary effect of condensed tannins within the digestive tract is due to the formation of less digestible complexes with dietary proteins, rather than by inhibition of digestive enzymes. One mole of tannin is reported to bind 12 moles of protein (Chung et al., 1998). Tannins are known to affect the utilization of vitamins and minerals. Inclusion of tannic acid in the diet resulted in reduced contents of vitamin A in rat liver and interference with vitamin B₁₂ utilization. Tannins form insoluble complexes with divalent iron rendering it less absorbable. The major effect of dietary condensed tannins was attributable to their inhibition on food consumption or digestion but rather the efficiency with which the digested and absorbed nutrients were converted into new body substances (Butler and Rogler, 1992).

Yet, tannins have also been considered a health-promoting component in plant derived foods and beverage. For example, tannins have been shown to have anticarcinogenic and antimutagenic potential and antimicrobial properties (Amarowicz, 2007). The inhibitory effect of tannins in cancer formation has been well documented. Tannic acid and green tea polyphenols (GTP) offered protection against skin tumor induced by BPDE-2 (8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzopyrene) (Chung et al., 1998). Quercetin also shows potent anticarcinogenic activity against cancers of skin, colon and mammary gland in rodents (Chung et al., 1998). Gallic acid, caffeic acid, and chlorogenic acid also reduced the formation of some mutagens (Gensler et al., 1994). Ellagic acid has been reported to significantly inhibit cancer formation in the colon, oesophagus, liver, lung, tongue and skin of rats and mice in both *in vitro* and *in vivo* investigations. This compound has also been demonstrated to be a possible chemopreventive agent against human carcinogenesis. Miyamoto et al., (1993) showed the antitumour activity of several

oligomers of hydrolysable tannins, including agrimoniin, oenothien B and coriariin A, against sarcomas.

The average content of condensed tannins in CS meal varied from 2-5 g/Kg (Russo and Reggiani 2012a) depending from origin of the sample. For Matthaus and Zubr (2000) the winter cultivars have the highest content of tannins. Compared to the other oilseeds such as soybeans and sunflower the content of condensed tannins in CS seeds was higher. However it was, comparable with crambe (3 g/Kg) mustard (2 g/Kg) and rapeseed (4 g/kg). (Matthaus and Zubr, 2000). The total amount of tannins in CS seed was relatively low and, therefore, low nutritional interference can be expected. Moreover, the nocive effects of tannins are detected when they represent more than 1% of the administered meal. According to some studies (Kumar and Singh, 1984), tannins make meal less palatable when they are present as higher as 20 mg/g of D.M.

1.3.4 Exploitation of CS meal

Several studies have suggested that new markets have to be developed for co-products of biofuel production to make the biofuels economically competitive and sustainable as compared to fossil fuels (Subhadra and Edwards, 2011). Exploitation of by-products of biofuel production in high-value applications will help to add substantial value, to reduce the cost of biofuel and make biofuels competitive with fossil fuels. Many experiences on crops for biofuel purposes has demonstrated that the sustainability based on the input and output energy used and on economical basis will be profitable only if the remaining flours after oil extraction, can be utilized, for example, for animal feedstuff.

The biodiesel extraction by-products from CS seeds are high in protein (about 45%) and the energetic value of CS cake (MJ ME/kg DM) is 15.0 for ruminants, 14.0 for pigs and 8.0 for poultry (Colombini et al., 2012). A potential disadvantage of using CS meal in livestock diets is its content of compounds, such as GSLs, phytic acid, condensed tannins and sinapine, that can reduce CS meal nutritive value (Matthaus and Zubr, 2000). A requirement for absence of quantifiable amounts of CS in livestock rations was prescribed by an EU Directive (2002). However, a recent EU directive (2008) underlined a renewed interest in CS as oilseed crop because of an increasing demand for alternative low-input oilseed crops with the potential for use of the by-products in animal feeding. Hence, the recent directive deleted the requirement for absence of quantifiable amounts of CS in livestock rations. Again in September 2008, the US Food and Drug Administration's (FDA) Division of Animal Feeds provided clarification to States regarding the limited use

of CS oilseed meal as a commercial feed ingredient. Although FDA prefers a written request for distribution of CS meal, States may approve the limited use of CS meal for in State commerce. Similarly, the American Food and Drug Administration has recently raised the inclusion level of CS meal in feedlot beef cattle rations to 10% of DM (Schill, 2010).

From January 2008, the State Department of Agriculture is working with several Montana industries and FDA to generate documentation of what is needed to acquire GRAS (generally recognized as safe) status and feed certification for CS meal (Mc Vay and Lamb, 2008). Preliminary research in Montana utilizing CS meal for production of omega-3 enriched livestock products has been promising. In these early studies, Montana has utilized conservative levels of CS meal in the feeds. Current research is evaluating higher levels of CS meal to determine levels of CS that negatively impact performance or product quality. In Montana, the main industry is beef cattle production. In 2006, CS meal was evaluated on its acceptability, and potential to replace soybean meal (SBM) in finishing beef cattle. Steers were fed 4 and 9 percent (dry matter basis) of a high concentrate diet of either SBM or CS meal. There were no statistical differences in the performance or weight gain of cattle fed SBM or CS diets. Sensory evaluation of steaks did not reveal any detrimental effect on taste or consumer acceptability. The omega-3 content of the muscle was slightly elevated. In 2007, CS meal was fed at zero, 10, 20 and 30 percent of the finishing diets of steers. This diet was used to determine if there was a decrease in the performance of finishing cattle while using CS meal to replace corn and soybeans for protein and energy sources (Mc Vay and Lamb, 2008; Darrin Boss Northern Agricultural Research Centre, Havre, Montana). CS meal was first evaluated for production of omega-3 dairy in 2005. The goats were fed with diets containing 15% CS for 4 weeks. The content of omega-3 in the milk increased with increasing CS content in the feed. Moreover, the ratio of saturated FAs (unhealthy) to unsaturated FAs (healthy) increases in milk from goats fed with CS meal. CS also has positive impacts on health, coat, and milk volume (Szumacher-Strabel, 2011). Hurtaud and Peyraud (2007) added CS cake in feed for dairy cows. The use of CS led to significant changes in milk fat composition and yield, and resulted in softer and probably more spreadable butter. Therefore it is advisable to keep CS meal below a threshold of 2 kg/copper die to limit certain adverse effects such as a decrease in milk fat content or an increase in the concentration of potentially unhealthy *trans* C:18:1 isomers. The US Egg and Poultry Association provided supplemental funding to analyze CS meal as an ingredient for

production of omega-3-rich eggs or broiler meat. CS cake seems to be a suitable feed ingredient for laying hens and broiler. CS inclusion adds protein and sulphur amino acids to feed and can replace, at least partly soybean meal. In addition, the high omega-3 FA content of CS ameliorates eggs and broiler meat's FA profile, especially ω -6/ ω -3 ratio without significant effects on eggs' sensory quality or detrimental effects on hens' and broilers' health or performances (Valkonen et al., 2007). The feeding trials conducted with CS meal in poultry were conflicting. Impaired feed conversion and decreased feed intake during the starter phase in birds fed with CS meal has been reported (Pekel et al., 2009). However, Frame et al. (2007) observed no significant differences in final weight, weight gain or feed conversion between feeding diets with 10% CS meal. Further studies are needed to determine the effectiveness concentrations of CS meal that would achieve beneficial properties in eggs and meat products without adversely affecting the production performance of birds. (Aziza et al., 2010). Salminen et al (2006) have studied about the use of residues of CS meal with the aim to determine if bioactive substances in them could be exploited as potential food ingredients in developing functional meat products. They concluded that CS meal was an excellent antioxidant toward oxidation of meat lipids. The responsible of this activity are sinapic acid and its derivative, and tocopherols. The antioxidant properties of CS meal are also enhanced by flavonols.

Series of experiments were carried out to assess the exploitation of CS cake in human nutrition. The development of food products was designed to evaluate the applicability of CS meal as ingredient in bread (Zubr, 2010). The bread was produced from wheat and rye flour. The proportion of CS was 5-10 % w/w of the flour used. The final products were characterised by a distinct smell and taste originating from CS. The intensity of the sensory effects was proportional to the amount of the ingredient added. The bread enriched with CS in various modifications is still on the market in Denmark.

The research conducted by Reddy et al., (2012) showed that CS meal can be potentially used for fiber, thermoplastics and other applications. CS meal grafted with various acrylates showed good thermoplasticity and ability to be compression molded into films with good dry and wet tensile properties.

1.4 Objectives

The studies reported in this thesis were performed in order to evaluate CS biodiesel by-product as ingredient in animal feed formulations.

The different objectives can be summarized as follows:

1. Study of protein content in nine genotypes of CS in a experimental field set up in Casazza (BG) in order to identify the genotype with highest protein content.
2. Study of quality of CS seed storage protein by using electrophoretic technique (SDS-PAGE).
3. Amino acid (AA) analyses of CS protein, by HPLC (high performance liquid chromatography) analysis after acid hydrolysis of extract protein. Focusing attention on essential AA content in order to determine the biological value of CS protein.
4. Determination of chemical composition of CS meal using official methods (AOAC,1995)
5. Determination of *in vitro* protein rumen degradability and protein degradation rates.
6. Study of levels of anti-nutritional compounds: GSLs, sinapine, phytic acid and condensed tannins.

2. Materials and Methods

2.1 CS protein analyses

2.1.1 Plant material

A total of nine genotypes of CS, different by origin, were used. Calena (Germany), Ligena (Germany), Cam 40 (unknown), Cam 45 (Russia) Cam 46 (unknown), Cam 172 (Russia), Cam 180 (Germany), FF006 (Austria), FF084 (Austria). A field experiment was carried out at Casazza (BG) in two years of cultivation and two sowing seasons: autumn (2008-2009; 2009-2010) and spring (2009; 2010). The field was located at 450 m above sea level in a hilly area previously utilized as permanent pasture.

2.1.2 Meal protein content

CS seeds were defatted with cold acetone and protein assayed according to the protocol developed by IBBA (Istituto di Biologia e Biotecnologia Agraria)-CNR (Consiglio Nazionale delle Ricerche), using the Plant Total Protein Extraction Kits (Sigma-aldrich, Milano, Italy) for extraction and Quantum Bicinchoninic Acid Protein Assay (EuroClone, Italy) for protein content determination. The Plant Total Protein Extraction Kits kit includes two reagents, a plant specific protease inhibitor cocktail and a chaotropic reagent with increased solubilizing power to extract more hydrophobic proteins.

2.1.2.1. SDS-PAGE of protein extracts.

Total seed proteins were extracted from defatted CS and Canola flours as described above. Equal amounts of protein (10 μ g) were applied on a 4 to 12% gradient SDS-polyacrilamide gel (Laemmli, 1970), and after electrophoresis the gel was stained with Coomassie brilliant blue.

2.1.2.3 Extraction and Fractionation of proteins

Proteins were extracted and fractionated from the defatted seed, based on the Osborne method as described by Ju et al., (2001). Water soluble protein (albumin) was extracted by stirring the dispersion of the defatted seed (0.1 g) in deionized water (1.5 ml) for 2 h at ambient temperature. The dispersion was then centrifuged at 15000 rpm (model T1 15A61 1015, HIMAC CT 15 RE VWR) for 20 min to separate supernatant containing the extracted albumin from residue. After water extraction, salt soluble protein (globulin) was extracted from the residue using 1 M sodium chloride solution (1.5 ml) by stirring the dispersion for 2 h at ambient temperature. The dispersion was then centrifuged at 15000 rpm for 20 min to obtain supernatant containing the globular protein fraction. Alkali-

soluble protein (glutelin) was extracted by adding 1.5 ml of 1M sodium hydroxide into the residue and stirring for 2 h at ambient temperature. The extracted glutelin in the supernatant was separated by centrifugation at 15000 rpm for 20 min. The residue after extraction from each solution was washed twice using a small portion of deionized water to collect the residual protein fractions from the residue. The washings and the first extract were combined for each fraction. Albumin, globulin and glutelin were precipitated, using 20% trichloroacetic acid, overnight. After centrifugation 15000 rpm for 20 min at 4°C, the residues were washed twice, and resuspended in 0.5 M sodium phosphate solution pH 7 with a plant specific protease inhibitor cocktail. Protein content of each fraction was determined using the protein assay above described.

2.1.3 Amino-acid composition

Total protein were extracted by Plant Total Protein Extraction Kit (Sigma-aldrich, Milano, Italy). Fifty μ l of sample were subjected to acid hydrolysis in 3M mercaptoethansulfonic acid and 0,2% Na azide at 110 °C for 16 h. Hydrolyzed samples were then diluted ten times before amino acid analysis. The amino acid composition was determined by HPLC analysis of the o-phthalaldehyde (OPA) derivatives according to Reggiani et al., (2000). The separation of OPA derivatives was performed at a flow rate of 0,8 ml min⁻¹ on a 150 x 4.6 mm Water Spherisorb ODS-2 3 μ reverse phase column (Grace Davison Discovery Sciences, Sedriano, Italy). Two mobile phase were used: A) 100 mM Na-acetate (pH 7.13) – tetrahydrofuran (99.5:0,5 v/v); B) methanol. Phase B was maintained at 5% v/v for 2 min and gradually increased to 50% (v/v) in 37 min, to 100% (v/v) in 3 min, and then returned to 5% (v/v) in 2 min. Proline was assayed in hydrolyzed samples by the acid ninhydrin method (Bates et al., 1973).

2.1.4 *In vitro* rumen protein degradability

Rumen protein degradability was determined on a single sample for CS genotype in a rumen in vitro system treated with hydrazine and chloramphenicol to inhibit microbial uptake of protein degradation products (ammonia, free amino acids and oligopeptides) as reported by Colombini et al (2011) using OPA colorimetric absorbance (OPA-C) at 340 nm to determine the content of amino acids oligopeptides or OPA fluorimetric (OPA-F) fluorescence to determine the content of free amino acid derived from samples protein degradation during incubation. Ammonia concentration was determined by phenol-hypochlorite assay. The inoculum was prepared from ruminal fluid obtained from 2

lactating Holstein Fresian donor cows. Care and general maintenance of the animals was conducted as outlined by the guidelines of the University of Wisconsin Institutional Animal Care and Use Committee. Cows were fed a diet containing alfalfa and corn silages plus a concentrate mixture of rolled high moisture corn, soybean meal, vitamins and minerals. Each sample was analyzed in 2 incubation runs. Degradation rate was determined using the integrated Michaelis-Menten model (Segal, 1976) as described by Broderick and Clayton (1992). Rates were adjusted for the N fractions already degraded at the beginning of the incubation and composed of ammonia, free amino acids and small peptides. Rumen-undegraded protein (%) (RUP) was calculated by applying 2 different rates of passage for insoluble and soluble N as described by Colombini et al (2011).

Statistical Analysis for digestibility protein data was carried out using the mixed procedure of the Statistical Analysis Systems (SAS Institute, 1999-2000). For the CS meal *in vitro* data, the model included the genotype as the main effect and the incubation run as a random effect. Estimates of least squares means are reported; separation of least squares means was conducted at $\alpha=0,05$ using the PDIFF option in the LSMEANS statement.

2.2 Chemical composition of CS meal

Seed meals from CS genotypes were obtained from 100 g of dry seeds after oil extraction using a Soxhlet apparatus (Carlo Erba; Italy) according to the official method (AOAC, 1995). Seed and meal samples were ground through a 1 mm screen (Pulverisette, Fritsch Idar-Oberstein Germany). Samples were chemically analysed for DM (method 945.15, AOAC, 1995), ash (method 942.05, AOAC, 1995), ether extract (method 920.29 AOAC 1995), neutral detergent fiber corrected for the insoluble ash and with the addition of α - amylase (NDF) (Mertens, 2002) and acid detergent fiber (ADF) (Van Soest et al., 1991) using the Ankom 200 fiber apparatus (ANKOM Technology Corp., Fairport, NY, USA), acid detergent lignin (ADL) (Van Soest, 1963) and N solubility (Licitra et al., 1996). The proportion of total N supposed to be already degraded at t=0 (ammonia, free aminoacids and oligopeptides) was determined on all proteins as reported by Colombini et al., (2011). The energy value of meals was predicted using the NRC (2001) equation and expressed as MJ of net energy of lactation (NE_1) calculated for intake at three times maintenance (NE_13x).

2.3 CS anti-nutritive analyses

2.3.1 Plant material

CS seeds were sown in spring in fields near Firenze, Italy. Twelve CS genotypes different by origin were used: Calena (Germany), Cam120 (Poland), Cam180 (Germany), D11851 (Italy), FF006 (Austria), Ames28372 (USA), Cam31 (Poland), Cam37 (Russia), Cam173 (Russia), D9952 (Unknown).

2.3.2 Extraction and separation of GSLs

2.3.2.1 Analytical procedures

CS seeds were defatted with cold acetone, GSLs were extracted with hot 70% ethanol for 3 h and the samples were then centrifuged for 15 min at 13,000 g. Five hundred μL of ethanol extract were adsorbed onto a small DEAE-Sephadex A-25 column in formate form (100 mg). The column was then washed twice with 1 mL of sodium acetate buffer (20 mM, pH 4.0). Desulfation of GSLs was obtained by 50 μL of sulfatase (500 U) at 37°C overnight. Desulfo-GSLs were eluted from the column with 1 mL of water and dried at 65°C. The samples were resuspended in ethanol before GSLs analysis.

2.3.2.2 Thin layer chromatography procedure

Ethanol samples were loaded with a Linomat IV (Camag, Switzerland) on an HPTLC (high performance thin layer chromatography) RP18W plate (Merck, Germany). The plate dimensions were 100x200 mm and, by applying bands and interspaces of 5 mm, it was possible to load up to 18 samples each time. The plate was developed

in a horizontal HPTLC Developmental Chamber (Camag, Switzerland) and the mobile phase was acetonitrile/water (4:6, v/v 15 mL per plate). After the run, the absorbance (229 nm) of each lane was read into a densitometer TLC Scanner II (Camag, Switzerland) using desulfo-sinigrin as standard. GSL data were quantitated by a D-2000 integrator (Hitachi-Merck, Germany). Calibration line with different concentrations of desulfo-sinigrin was calculated by the least squares regression model and t-test for significance.

2.3.2.3 High-performance liquid chromatography procedure

GSLs were determined according to the method of Kraling et al., (1990) modified to improve the separation of CS GSLs. Desulfo-GSLs were separated by gradient HPLC and detection at 229 nm. A 150 mmx4.6 mm Waters Spherisorb ODS-2 (3 μm) was used for

separation. The mobile phase consisted of two eluents) water (HPLC-grade) acetonitrile. The flow rate was 0.7 mL min⁻¹. The program started with 95% A and 5% B for 2 min followed by a linear gradient over 25 min to 5% A and 95% B. This was held for 2 min before the program returned to 95% A and 5% B by a linear gradient of 1 min followed by at least 10 min equilibration.

2.3.3 Extraction and separation of Sinapine

The extraction of sinapine on defatted flours was achieved with 70% methanol as described by Cai and Arntfield (2001). The HPLC analysis for sinapine was performed according to Clausen et al. (1985). The separation of sample was performed at a flow rate of 275 µl/min in isocratic conditions on a 2,1mmx100mm Waters ODS phase column and using acetonitrile 13,5% and buffer acetate 10 mM pH 4 as mobile phase. Sinapine and sinapic acid were detected with an UV-detector at 330 nm and calculated using sinapine bisulphate extracted from *Sinapis alba L.* as a standard (Mailer et al., 2008).

2.3.4 Extraction and assay of Phytic Acid

Phytic acid was isolated from defatted flour using a modified acid extraction-iron precipitation method by DeBoland et al. (1975). The phosphorus content of the precipitate was determined, after acid digestion with sulfuric acid, colorimetrically according to Chen et al. (1956). Phytic acid was calculated by multiplying phytic acid phosphorus values by 3.55 (Raboy and Dickinson, 1984).

2.3.5 Extraction and assay of Condensed Tannins

The determination of condensed tannins was carried out as described by Butler et al. (1982). Tannins were extracted from defatted flour with 70% acetone, the samples evaporated to dryness and then resuspended in methanol. Condensed tannins were determined by the vanillin method using catechin as a standard (Mathäus and Zubr, 2000).

2.4 Statistical Analysis

All statistical analysis except for digestibility data was performed by SPSS version 11.5 software. Analysis of Variance (ANOVA) was applied to establish significant differences (P<0.01) between CS genotypes in the levels of protein and anti-nutritive compounds. Mean separation was performed using Duncan's test and referring to P≤0.05 probability level. Pearson's correlations between anti-nutritive compounds were also calculated.

3. Results and Discussions

3.1 CS meal: protein characterization

3.1.1 Meal protein content

Table 3.1 shows the protein content in nine genotypes of CS from experimental field set up in Casazza (BG). Data were obtained, from two different years of cultivation and two growing seasons (autumn and spring). The mean value of protein content (% of dry defatted flour weight) ranged from 30.7% (autumn sowing 2008) to 37.7% (spring sowing 2010). In autumn sowing, Ligena (35.0% in defatted flour) was the genotype with the highest protein content in both years. In general the protein content was higher in the seeds from spring sowing than from autumn sowing in both years of cultivation.

As evidenced by ANOVA analysis (**Table 3.2**) protein content resulted significantly different both for sowing season and year of cultivation but not among the genotype.

Probably, plants growing in spring sowing are exposed to higher temperature during seed filling thus allowing a better mineral nutrient absorption and utilization. Nitrogen and sulphur in soil and climatic conditions affect the protein content of CS meal (Zubr, 1997 and Urbaniak et al., 2008). Nitrogen is one of the most important factors in oilseed crops yield. A number of research projects have evaluated N requirements in CS. According to Zubr (1997), CS can be successfully grown at 100 kg N/ha. Nitrogen is incorporated into amino acid skeletons, and increasing N supply intensifies protein synthesis. Urbaniak et al., (2008) found that the protein content in some genotypes of CS increased from 22% to 30% when the N rate was increased from 0 to 120 kg/ha. Sulphur is an important macroelement and oil plants are usually very sulphur-demanding in terms of oil and protein contents. A deficit of sulphur inhibits the utilization of nitrogen from fertilizers and may increase the escape of nitrogen into the environment, especially via leaching of nitrates into the hydrosphere or volatilization into the atmosphere. Of course, sulphur is a component of the amino acids cysteine and methionine, which are required for proteosynthesis (Losak et al., 2010). Other parameters that influences protein content are high temperature and low rainfall during seed filling. Plants exposed to high temperature increase leaf transpiration and raise the absorption and the velocity of translocation of mineral salts (nitrogen, sulphur, etc..) into xylematic tubes.

Table 3.1: Meal protein content (% in defatted flour) in nine genotypes of CS from experimental field set up in Casazza (BG), in two different years of cultivation and two growing seasons: autumn and spring. The data are the mean (\pm S.E.) of 3 assays of 3 blocks for genotype.

Autumn sowing			Spring sowing	
	2008-2009	2009-2010	2009	2010
Protein % in defatted flour				
Calena	31.7 \pm 0.8 (c)	33.8 \pm 1.1 (ab)	37.0 \pm 0.5 (d)	39.8 \pm 0.3 (b)
Ligena	35.0 \pm 0.8 (d)	35.0 \pm 0.8 (b)	35.5 \pm 0.3 (cd)	36.8 \pm 0.8 (a)
Cam 40	29.0 \pm 0.5 (b)	34.3 \pm 0.7 (ab)	33.0 \pm 0.4 (a)	37.8 \pm 0.3 (a)
Cam 45	31.0 \pm 0.6 (c)	34.0 \pm 0.3 (ab)	34.9 \pm 0.3 (cd)	40.2 \pm 0.3 (b)
Cam 46	31.6 \pm 0.6 (c)	32.8 \pm 0.3 (ab)	34.4 \pm 0.4 (bc)	36.3 \pm 1.1 (a)
Cam 172	32.8 \pm 0.5 (d)	31.9 \pm 1.0 (a)	33.4 \pm 0.3 (ab)	36.7 \pm 1.1 (a)
Cam 180	35.0 \pm 0.5 (d)	32.0 \pm 0.3 (a)	35.1 \pm 0.5 (cd)	n.d
FF006	24.5 \pm 0.3 (a)	33.3 \pm 0.7 (ab)	32.4 \pm 0.3 (a)	37.2 \pm 0.4 (a)
FF084	25.9 \pm 0.5 (a)	33.4 \pm 0.3 (ab)	32.6 \pm 0.3 (a)	36.5 \pm 0.5 (a)
MEAN	30.7 \pm 1.3	33.4 \pm 0.4	34.3 \pm 0.5	37.7 \pm 0.5

Values with different letters are significant different at $P \leq 0.05$ (Duncan test)

Table 3.2: Mean square and F value from Analysis of Variance (ANOVA) for protein content in flours of nine genotypes of CS.

Effects	d.f.*	Mean square	F value
YEARS	1	72.69	8.37**
SOWING SEASON	1	128.43	18.36**
GENOTYPES	1	7.26	0.63

* d.f. = Degrees of freedom; ** = Significant at $P \leq 0.01$

In **Figure 3.1** the electrophoretical profiles of protein extracted from CS meal and canola meal are shown. In CS, the pattern shows two major bands: 27 kDa and 15 kDa. There are few data in literature about CS seed proteins. Thus, it was necessary a comparison with the protein pattern of Canola (a specie close to CS). As described in literature (Crouch and Sussex, 1981; Hoglund et al., 1991; Berot et al., 2005), the two main of storage proteins in rapeseed are the 2S albumin, napin and the 12S globulin, cruciferin. They represent 20 and 60% respectively of the total protein in mature seeds. Native cruciferin is composed of six subunits arranged as two trimers tied by salt bridges. Each subunit of this hexameric assembly is composed by acidic and basic polypeptides of 33 and 27 kDa bands in weight named α and β -cruciferin respectively linked with one disulfide bond (Adachi et al., 2003). In Canola profile 33 and 27 kDa bands correspond to α and β -cruciferin respectively, as reported by Hoglund et al. (1991). In CS profile there is a remarkable polypeptide(s) at 27 kDa which probably correspond to the cruciferin region (globulin). Also in CS, globulins represent at least 60% of total protein (**Figure 3.2**). Mature napins are highly basic proteins which comprise two polypeptide chains held together by two disulfide bonds (Berot et al., 2004). In Canola pattern, 16 and 15 kDa correspond to heavy chains of napin, 13 and 12 kDa correspond to light chains of napin as reported by Hoglund et al. (1991). In CS, there is a main band of 15 kDa in the same region of napin (albumins). Anyway the native structure of these storage protein of CS is still unknown.

Figure 3.1: SDS-PAGE profile of protein extract of CS and Canola. Black points indicate the major bands detected in canola protein profile.

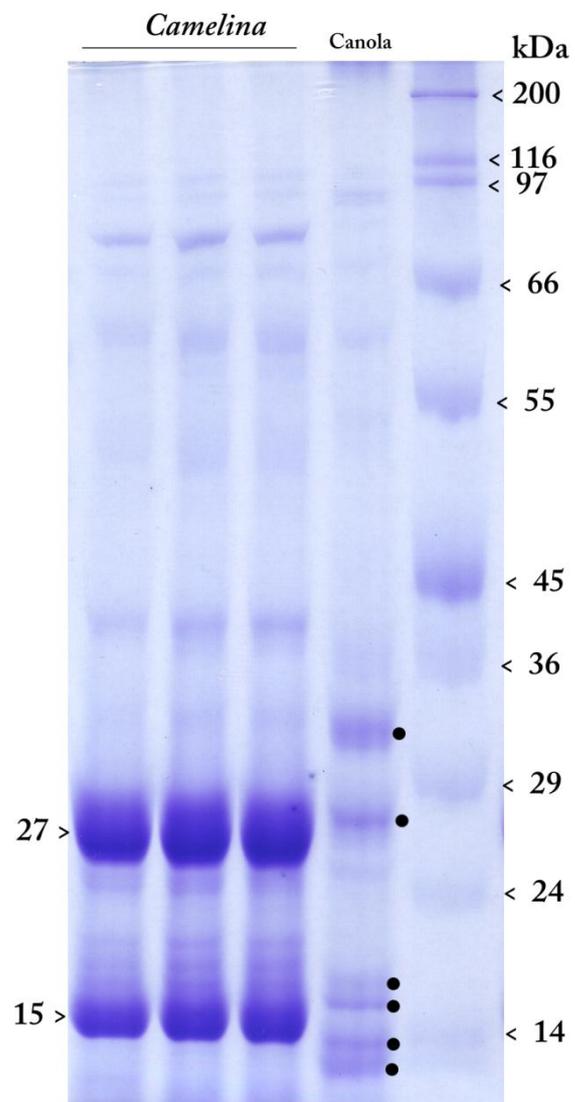
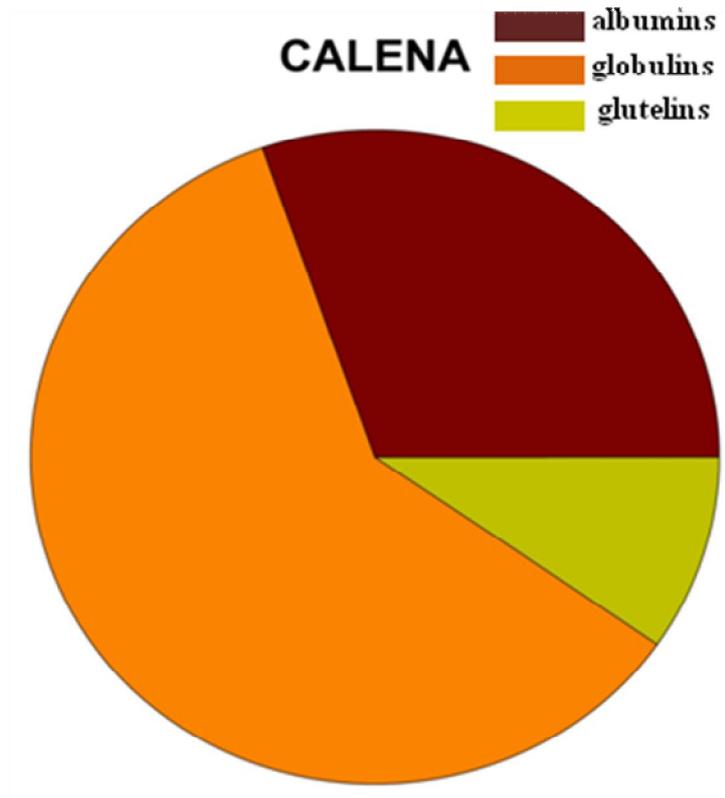


Figure 3.2: Proteins of CS divided in class of solubility.



3.1.2 Amino acids (AA) in CS proteins.

Table 3.3 shows the amino acid content in hydrolyzed protein of CS var. Calena. Asparagine and glutamine are deaminated by hydrolysis into aspartate and glutamate. The nutritional value of protein is evaluated by the profile of essential amino acids (AA). The protein of CS meal consisted of all AA including the ten essentials. Leucine with 69.1 g/Kg CP (crude protein) and valine (60.4 g/Kg CP) were predominant among the essential AA in CS meal. Plant storage protein are often poor in lysine and sulfurated AA. The content of the lysine was 45.1 g/Kg CP, while the content of sulfurated was methionine was 24.8 g/Kg CP and 19.0 g/Kg CP for methionine and cystine respectively. Among non-essentials AA, arginine was predominant with 82.3 g/Kg CP. Zubr (2003) compared the nutritional value of CS meal respect to other oilseed like rapeseed soybean and flaxseed. Rapeseed was higher in lysine (65 g/Kg C.P) respect to CS meal (45,1 g/Kg C.P), but was lower in arginine (67 in against 82.3 g/Kg C.P). Soybean protein was higher in aspartate (127 g/Kg C.P) and glutamate (190 g/Kg C.P), while the content of valine (32 g/Kg C.P) was lower than in CS meal (60.4 g/Kg C.P). Flaxseed protein was particularly high in arginine (110 g/Kg C.P) and glutamate (190 g/Kg C.P) while lysine (46 g/Kg C.P) and glycine (78 g/Kg C.P) were similar to CS meal. **Table 3.3** shows the AA scores of CS var. Calena. The AA score determines the effectiveness with which adsorbed dietary nitrogen can meet the indispensable AA requirement at the safe level of protein intake. This is achieved by a comparison of the content of the limiting amino acid in the protein with its content in the requirement pattern.

$$\text{Score} = \frac{\text{g of AA in test protein}}{\text{g of AA in requirement pattern}} \times 100$$

The test protein is determined by the FAO/WHO/UNU expert consultation held in Geneva (2002). For determination of biological value of flour the essential AA content is important. The lowest score determines the biological value of protein meal. Score of 100 indicates an equilibrated AA content while a lower score means that one or more AA are limiting. In the case of CS meal, lysine (45.1 g/Kg CP) results limiting because it shows a score of 98. Since this value is close to 100, CS proteins can be considered of good quality.

Table 3.3: Amino acid content (g/kg CP) and scores in proteins of CS var. Calena.

Amino acid	Reference*	Calena	SD	SCORE
Aspartate+Asparagine		90.6	5.5	
Glutamate+Glutamine		149.6	9.8	
Serine		59.7	2.5	
Glycine		60.6	1.9	
Alanine		59.9	2.8	
Arginine		82.3	4.1	
Proline		60.9	3.2	
Histidine	15	42.2	4.2	281
Threonine	23	28.6	1.8	124
Valine	39	60.4	2.2	155
Methionine	16	24.8	1.0	155
Cystine	6	19.0	0.8	317
Tryptophan	6	13.0	0.3	217
Phenylalanine	30**	51.7	3.5	293
Tyrosine		36.2	3.1	
Isoleucine	30	46.3	2.7	154
Leucine	59	69.1	0.7	117
Lysine	46	45.1	3.2	98

*= As determined by the FAO/WHO/UNU expert consultation held in Geneve (2002); **= Phenylalanine+tyrosine

Table 3.4 shows the biological value and respective limiting AA of CS and other plants used in animal feed: selected oilseed like rapeseed and sunflower, cereals like oat, wheat and corn and legumes like soybean and bean. Rapeseed has the highest biological value with the good and balanced AA profile. Other species results in one or two limiting AA thus influencing the nutritional value of protein. CS protein is limiting in lysine like hempseed and cereals. As reported in **table 3.4**, cereals present proteins of low quality in comparison with those of CS . In general, legumes present methionine as limiting AA.

Table 3.4: Biological value and respective limiting amino acid of CS in comparison with the main plants used in animal feed.

FOOD	BIOLOGICAL VALUE	Limiting Amino acid
<i>Camelina sativa</i>	98	Lys
Rapeseed*	100	balanced
Flaxseed*	90	Met; Lys
Hempseed*	91	Lys
Soybean***	86	Met
Black Bean***	74	Met
Pea***	73	Met
Peanut***	55	Met
Oat**	53	Lys
Corn**	51	Lys; Trp
Wheat**	44	Lys
Sunflower**	39	Lys
Millet**	28	Lys; Thr

*= Data from CNR-IBBA (Istituto di Biologia e Biotecnologia Agraria) Milano

**= Data from Sarwar (1997)

***= Data from Sarwar and Peace (1994).

3.1.3 CS meal: composition and rumen protein degradability.

In **Table 3.5** the chemical composition of CS meals is reported. Average EE (ether extract), and ADF (acid detergent fibre, part of plant cell wall soluble in solutions at pH=4) contents of CS meal were 59.0 and 272 g/Kg DM (dry matter) respectively. Fibre content calculated as NDF (neutral detergent fibre, part of plant cell wall soluble in solutions at pH=7) was about 370 g/Kg DM. Calena was the genotype with highest fibre content (422 g/Kg DM) while Cam 46 had the lowest fibre content (328 g/Kg DM). Lignin content was about 59.3 g/Kg DM, and as resulted for fibre content, calena was the genotype with highest lignin content (67.6 g/Kg DM) and Cam 46 the lowest (39.2 g/Kg DM). Fibre content in CS meal (40% DM) was higher than that reported by Zubr (2010). CS presents a fibre content higher than rapeseed (30% DM) and flaxseed (10% DM). Lignin content in CS (6.5% DM) was identical to flaxseed (6.5% DM) (Zubr, 2010) and lower than rapeseed (8.6%) (Colombini et al., 2011).

The rates of rumen protein degradation (mg N/h) and the Ruminally Undegradable Protein (RUP) (g/Kg CP) obtained by the OPA-C assay are reported in **Table 3.6**. There are significant differences among the genotypes for degradation rates and estimated RUP values. The average degradation rate was 0.170 mg N/h. The highest rates were obtained for Cam 40 and Cam 45. The RUP mean value was 322 g/Kg C.P. The average protein content of CS meal (34% in defatted flour) was slightly lower than the value (40% in defatted meal) reported for soybean by NRC (Nutrient Requirement of Dairy Cattle) (2001).

In order to optimize the use of CS meal and to avoid the formulation of imbalanced rations for ruminants that may impair animal performance the estimation of ruminal protein degradation rates and RUP content are necessary. Differences for protein degradation rates and RUP observed in this study can be partially explained by the difference among genotypes in NDFIP (protein bound to NDF fraction) content in CS meal. It has to be emphasized that the CS meal samples were prepared in the same laboratory by the same operator; consequently, extraction method effects are limited. Some CS meal samples were not different from rapeseed meal and the RUP value of rapeseed was between the values reported by “*in vivo*” studies of Brito et al., (2007) (340 g/Kg) and Satter (1986) (230 g/Kg). The overall rumen protein degradation rate (0.150 mg N/h) was higher than the rates reported by NRC (2001) for rapeseed (0.104 mg N/h) and solvent soybean meal (0.094 mg N/h). These differences are probably due to differences in the protocol for

degradation rates: the NRC model is based on an “*in situ*” method, which has several limitations (such as porosity of bag material, ratio of sample weight-to-bag surface area, particle size of sample and bacterial attachment to feed residues) that can affect evaluation of protein degradation. Degradation for solvent soybean meal and expeller soybean meal measured “*in vivo*” by Reynal and Broderick (2003) were higher than that reported by NRC (2001) and were closer to the values determined on the same samples by the “*in vitro*” reported by Colombini et al., (2011)

Table 3.5: Chemical composition (g/Kg DM) and calculated energy value (MJ of NE_l/Kg DM) of CS meal genotypes.

Genotype	Ash	EE	NDF	ADF	ADL	NDFIP	ADFIP	NE _l 3x
Calena	58.5	93.3	422	300	67.6	60.5	38.5	8.07
Ligena	58.9	76.3	393	279	65.2	65.5	35.7	8.02
Cam 40	64.5	63.6	368	264	52.7	62.9	32.5	8.01
Cam 45	59.3	67.5	363	271	62.0	61.4	31.9	8.03
Cam 46	66.1	41.7	328	274	39.2	60.2	31.2	8.08
Cam 172	62.0	57.4	360	267	67.1	59.5	38.1	7.70
Cam 180	66.7	41.7	405	298	62.8	59.5	35.0	7.44
FF006	62.4	47.5	353	266	65.5	59.3	38.7	7.71
FF084	66.2	42.4	363	229	58.4	56.7	30.5	7.59
MEAN	62.7	59	372.8	272	60.1	60.6	34.7	7.9
SE	1.1	5.6	9.6	7	3	0.8	1.1	0.08

NE_l: Net energy of lactation; EE: Ether extract; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; ADL: Acid detergent lignin; NDFIP: protein bound to NDF fraction; ADFIP: protein bound to ADF fraction

Table 3.6: *In vitro* ruminal protein degradability of CS meals determined by the OPA colorimetric assay.

Genotype	N g/Kg DM	Soluble N g/Kg DM	Adj.Rate * (mgN/h)	RUP** (g/Kg CP)
Calena	68.1	27.6	0.136	329(ab)
Ligena	71.2	32.0	0.129(c)	356(a)
Cam 40	71.0	27.5	0.175(ab)	273(bc)
Cam 45	71.2	27.9	0.190(a)	255(c)
Cam 46	76.2	33.8	0.135(c)	348(a)
Cam 172	70.4	27.7	0.140(bc)	320(ab)
Cam 180	77.4	30.2	0.147(bc)	323(ab)
FF006	76.2	33.7	0.123(c)	364(a)
FF084	73.1	28.9	0.135(c)	332(ab)
MEAN	72.8	29.9	0.145	322.2
SE			0.0011	18.7
P Genotype	ns	ns	0.01	0.01

*= Protein degradation rate adjusted for proportion of total N present as ammonia, free amino acids and oligopeptides at the start of the incubation.

**= RUP, ruminal undegradable protein (Colombini et al., 2011).

a,b,c LS-MEANS within the same column with different superscript are significantly different (P<0.05)

3.2 CS meal: anti-nutritive compounds

A limitation in the use of oilseed by-products in diets is the presence of anti-nutritive compounds. Glucosinolates (GSLs) and sinapine were taken into account because they are typical of Brassicaceae plants, while phytic acid and condensed tannins are widespread in plant kingdom. **Table 3.7** shows ANOVA for GSLs, phytic acid, sinapine and condensed tannins for twelve genotypes of CS. ANOVA showed highly significant genotype variation (P<0.01) for all the antinutritive compounds considered.

In **Table 3.8**, the levels of anti-nutritive compounds for twelve CS accessions are shown. As can be seen, GSL content ranged from 15.2 to 24.6 mmol/Kg (DM), with most

genotypes with less than 20 mmol/Kg DM. Significant differences between genotypes were put in evidence by Duncan's range test, with the group marked with the letter (a) exhibiting GSL contents below the general mean.

Table 3.7: Mean square and F value from Analysis of Variance (ANOVA) for anti-nutritive compound contents in flours of twelve genotypes of CS.

Chemical compound	d.f. ^a	Mean square	F value
GSLs	11	21.49	23.00** ^b
Phytic acid	11	10.66	11.35**
Sinapine	11	0.61	57.29**
Condensed tannins	11	2.47	84.02**

^a d.f. = Degrees of freedom

^b ** = Significant at $P \leq 0.01$

Table 3.8: Anti-nutritive compounds in different genotypes of CS.

Genotype	GSLs*	Phytic acid**	Sinapine**	Condensed Tannins**
Calena	19.9 (b)	25.4 (a)	2.28 (de)	4.00 (d)
Ligena	19.8 (b)	27.8 (b)	1.87 (b)	1.92 (a)
Cam120	16.3 (a)	31.6 (ef)	2.38 (de)	2.85 (b)
Cam180	20.1 (b)	32.3 (f)	1.85 (b)	2.09 (a)
D11851	24.6 (c)	29.4 (bc)	1.58 (a)	3.41 (c)
FF006	15.2 (a)	29.4 (bc)	1.94 (b)	3.92 (d)
Ames28372	15.2 (a)	30.1 (cde)	2.93 (f)	4.39 (e)
Cam31	18.7 (b)	29.7 (cd)	2.82 (f)	3.96 (d)
Cam37	16.5 (a)	31.3 (def)	2.15 (d)	2.17 (a)
Cam76	19.2 (b)	31.6 (ef)	2.47 (f)	3.70 (d)
Cam173	16.9 (a)	30.9 (cdef)	2.79 (f)	2.82 (b)
D9952	19.7 (b)	29.3 (bc)	2.83 (f)	1.96 (a)
Mean	18.5	29.9	2.32	3.10
SE	0.8	0.6	0.14	0.27

* data expressed as mmol/Kg;

** data expressed as g/Kg; Means with different letters in parentheses with the same row differ significantly by Duncan's range test ($P \leq 0.05$)

Differences among genotypes were also observed on the GSL pattern (**Figure 3.3**). In CS, three main GSLs were identified named GSL1 (9-methyl-sulfinyl-nonyl-GSL), GSL2 (glucocamelinin, 10-methyl-sulfinyl-decyl-GSL) and GSL3 (11-methyl-sulfinyl-undecyl-GSL) (Russo and Reggiani, 2012b). In all genotypes, GSL2 represented the most abundant GSL being between 50%-60% (**Figure 3.3**). The content of the other 2 species, GSL1 and GSL3, exhibited differences among genotypes. Six genotypes showed levels of GSL1 higher than that of GSL3 (Calena, Ligena, Cam120, Cam180, D11851 and FF006). In 5 genotypes, GSL1 and GSL3 levels were similar (Cam31, Cam37, Cam76, Cam-173 and D9952). This different GSL pattern was suggested to be associated to winter genotypes or wild *Camelina* like *C. microcarpa* or *C. pilosa* (Schuster and Friedt, 1998). In AMES 28372, GSL3 was higher than GSL1. The climatic and soil conditions affects the content of GSLs (Matthaus and Zubr, 2000; Schuster and Friedt, 1998). Expecially sulfur in soil is a

determinant of the concentration of total GSLs in plant organs (Omirou et al., 2009). The 12 genotypes of different origin were grown in the same location in order to avoid variations due to the environment or soil. . In these conditions, 15.2 mmol/Kg DM was the lowest concentration of GSLs observed among genotypes (**Table 3.8**). Although CS has a relatively low concentration of GSLs in flour, this is higher than that required by EFSA (2008). Breeding in rapeseed reduced GSL content in meal from 50-150 mmol/Kg DM to less than 2 mmol/Kg DM (Sang and Salisbury, 1988; Bell et al., 1991).

In CS, the objective of low GSLs would be closer to reach and the different GSL pattern among genotypes might help (**Figure 3.2**). Nevertheless, some studies showed the use of CS meal in animal diet as it is (Hurthaud and Peyraud, 2007; Pekel et al., 2009; Aziza et al., 2010).

Figure 3.3: Distribution (%) of GSL1 (black bar), GSL 2 (light gray bar) and GSL 3 (dark gray bar) on total GSL content in twelve CS genotypes.

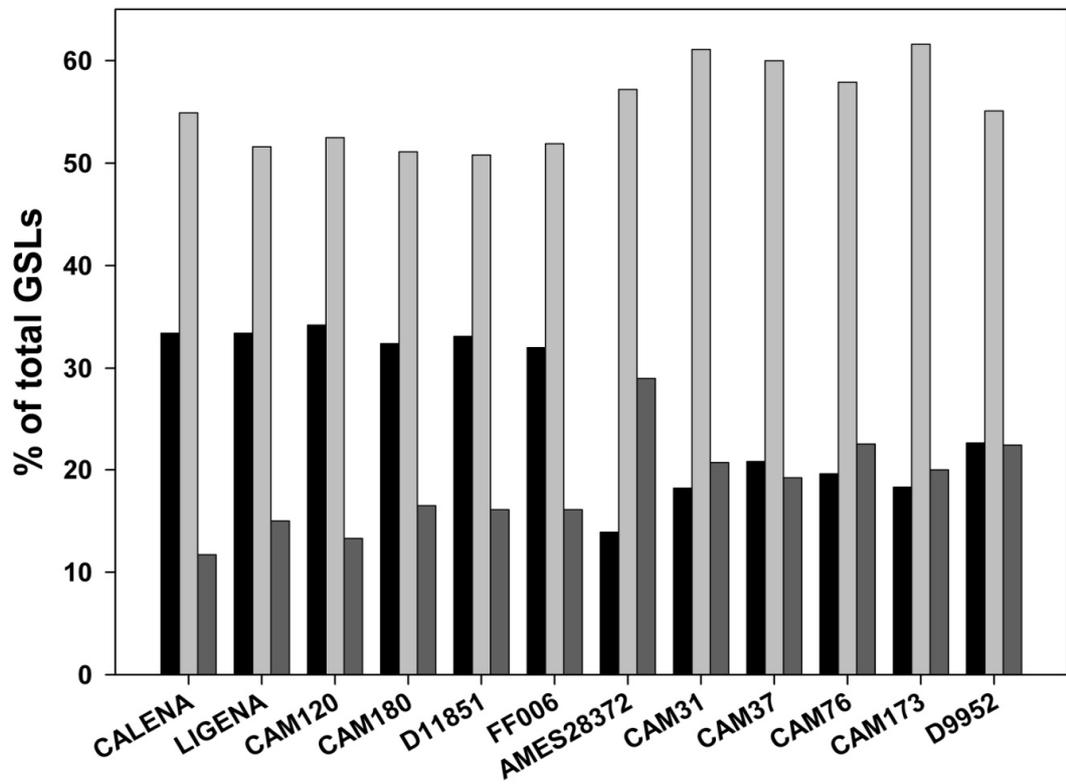


Table 3.9: Pearson correlation coefficients (r) between the different anti-nutritive compounds in twelve genotypes of CS.

	GSLs	Phytic acid	Sinapine	Condensed tannins
GSLs	1	-0.103	-0.421**	-0.181
Phytic acid		1	0.089	-0.238
Sinapine			1	0.249
Condensed tannins				1

**Correlation is significant at the level 0.01 level

The content of phytic acid ranged from 25.4 to 32.3 mg/Kg DM (**Table 3.8**). Significant differences between genotypes were observed and, in particular, Calena genotype resulted different and lower in phytic acid than the other genotypes. The content in this genotype is just higher than the content of phytic acid in soybeans (Kwanyuen and Burton, 2005), which flour is one the main ingredients in animal diet.

The sinapine content ranged from 1.58 g/Kg (D11851) to 2.93 g/Kg (Ames 28372), with a general mean of 2.32 g/Kg DM (**Table 3.8**). This sinapine content is similar to that reported for CS by Matthäus (1997) and can be considered low in comparison with other members of Brassicaceae (rapeseed, mustard).

The variation of the condensed tannin content in different CS genotypes was quite high. In **Table 3.8**, the contents varied from 1.92 g/Kg to 4.39 g/Kg of defatted flour. Matthäus (1997) found that CS genotypes contained from 1.5 to 3 g condensed tannins per Kg of seed. Considering that defatting seeds results in a concentration of condensed tannins, our data can be considered similar to that previously reported. These levels are no toxic in animal diets since they are below 1% DM (Singleton et al., 1981).

In **Table 3.9** is shown the correlation matrix calculated for the different anti-nutritional compounds. In this table, Pearson correlation coefficients are given as a measure of linearity between two class of compounds. From **Table 3.9**, it becomes clear that there is a significant inverse correlation ($P < 0.0.1$) between GSLs and sinapine.

For its bitter taste, the role of sinapine in plants is probably to make flours less palatable to animals. GSL degradation by animal metabolism instead affects animal growth, reproductive performance as well as intake and palatability of fodder (Matthaus and Angelini, 2005). It might be that sinapine and GSLs are part of the same defense mechanism against herbivores. This would explain the inverse correlation here observed

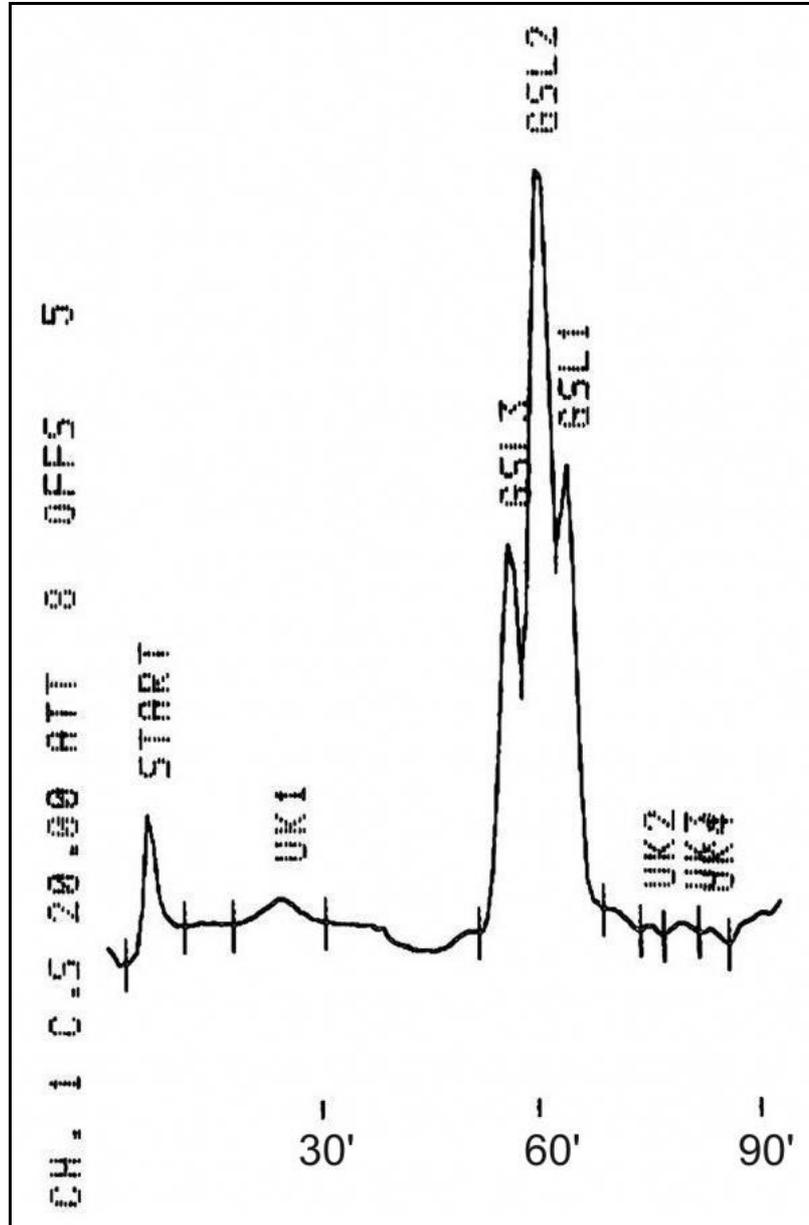
between these compounds so that the decrease of one is compensated by the increase of the other.

3.2.1 Rapid separation of seed GSLs from CS by thin layer chromatography (TLC)

Since the use of CS flour in animal diets appears to be limited by the presence of GSLs, a method by TLC that saves time and solvent was developed for screening CS genotypes.

Traditional separation of GSLs by HPLC (high performances liquid chromatography) occurs with a linear acetonitrile gradient on a C18 column in approximately 25-30 min and the system is generally ready for a new run after 35-40 min. In CS, three long chain GSLs are described (Matthaus and Angelini 2005; Russo and Reggiani, 2012b). Here, it was used a HPTLC (high performances thin layer chromatography) plate which have an optimized silica 60 layer with 5-6 μm particles modified with the same phase used for HPLC separation (RP-18). Separation of GSLs from CS Calena was achieved by TLC (**figure 3.4**). The 3 main GSLs migrated on an HPTLC RP18W plate with R_f of 0.58, 0.63 and 0.68. GSL1, GSL2 and GSL3 represented 28.7%, 44.4% and 22.6% of total GSLs, respectively. Traces of other GSLs were only 4%. A similar peak distribution was observed for all the six genotypes (*data not shown*).

Figure 3.4: Chromatogram of glucosinolates of CS var. Calena. GSL1: 9-methylsulfinyl-nonyl GSL; GSL2: 10-methyl-sulfinyl- decyl GSL; GSL3: 11-methyl-sulfinyl-undecyl GSL; UK, unknown.



A comparison was made between the HPLC and TLC procedures for the quantitation of total GSLs in six genotypes of CS (**Table 3.10**). The content of GSLs in the six genotypes ranged from 26 to 29.4 mmol/kg of defatted flour. There was no difference between those values obtained by TLC and those determined by HPLC analysis. The linearity and precision of the TLC method were evaluated within the range of interest.

The calibration curve of standard desulfo-sinigrin was obtained by plotting peak area against the different concentration (**Figure 3.5**). Linear regression was obtained within 0 to 400 nmol of desulfo-sinigrin with a regression coefficient (R^2) of 0.989 and an equation of $y = 8884 x$ ($P < 0.001$). The limit of detection was determined empirically by testing dilutions of desulfosinigrin standard solutions until no peak could be observed at 229nm; the limit was 5 nmol.

CS oil and residue of the oil-pressing process are strongly linked such that the success of the oil crop depends on the utilization of both products. According to European and North American guidelines (Schill, 2010) the possibility to use CS cakes or flours depends from the content of GSLs, it is important to have a fast and reliable method to evaluate these substances. Furthermore, evaluation of genotypic variation for GSL content is considered to be of primary importance for breeding CS plants low in GSLs. This TLC procedure for the screening of large plant populations is rapid and precise. The TLC protocol allows to load up to 18 samples per TLC plate every 90 min, whereas HPLC allows 2 samples every 90 min. This is a great saving in time and, economically significant, also in solvent.

Furthermore, the TLC procedure identified the same CS long-chain GSLs as HPLC (**Figure 3.4**) and the results obtained by both methods were similar (**Table 3.10**). The TLC method was linear in the range of GSL concentrations normally observed in CS extracts (25-30 nmol/mg of flour). Therefore, TLC represents a fast and economic alternative to HPLC for the determination of CS GSLs.

Figure 3.5: Calibration curve for GSLs: y, densitometer response (peak area); x, amount of desulfosinigrin loaded on plate (nmol). $R^2=0.989$.

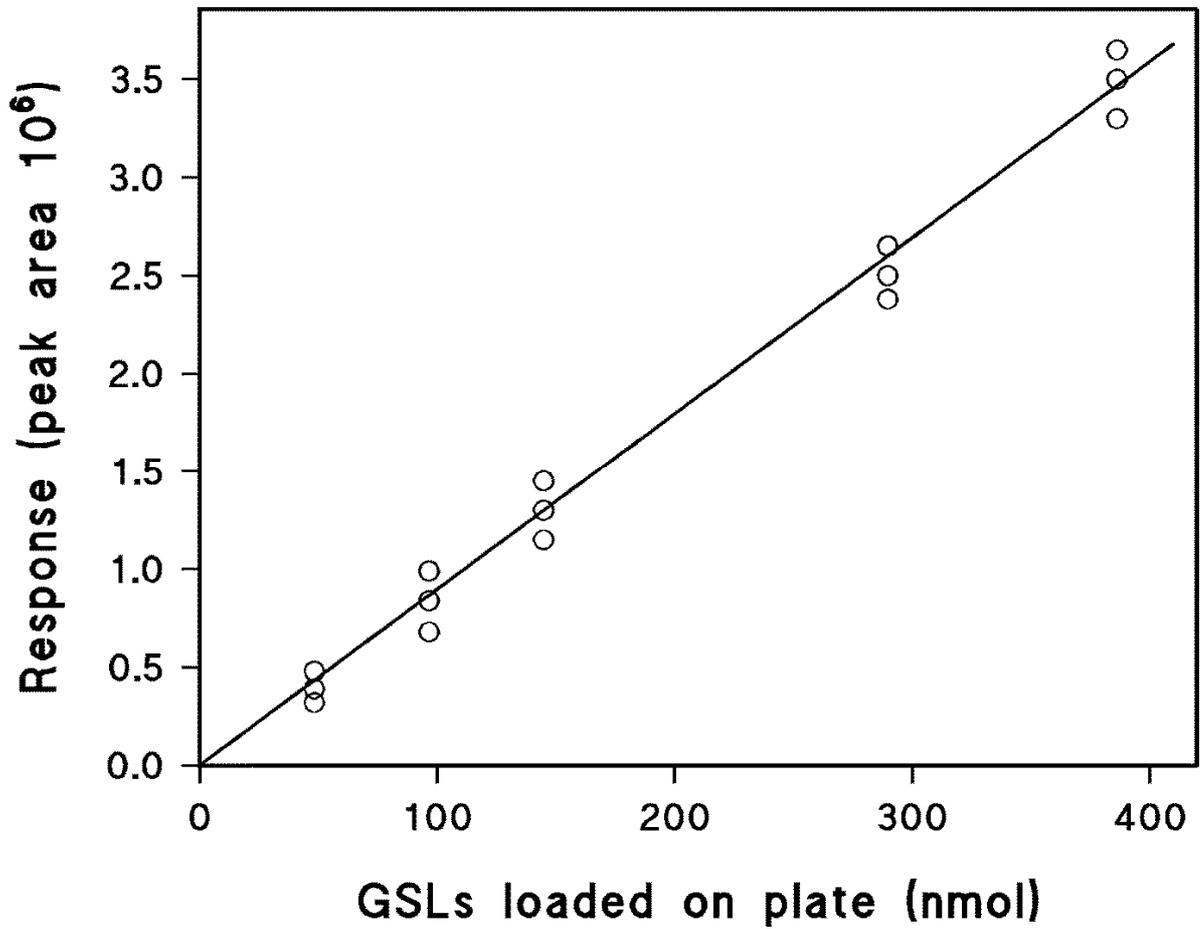


Table 3.10: Glucosinolates content (GSL1+GSL2+GSL3) in flour of six CS genotypes determined by high-performance-liquid chromatography (HPLC) and thin layer chromatography methods (TLC).

Genotype	HPLC	TLC
Calena	26.7±1.6	27.0±1.7
Ligena	29.0±1.2	29.4±1.6
Cam 40	26.2±0.8	26.4±0.8
Cam 172	28.2±1.0	28.5±2.8
FF006	29.2±1.1	28.4±2.2
FF084	27.3±1.0	28.7±2.1

4. Conclusions

The commercial transport industry has started evaluating the use of biofuels as a means of reducing the carbon footprint of vehicle transport. Biodiesel is an environmentally attractive alternative to conventional petroleum diesel fuel with the advantage to reduce GHG (greenhouse gas) emissions in the transportation sector. Crucifer oilseed plant *Camelina sativa* (CS) represents an alternative to rapeseed or flaxseed as biodiesel feedstock.

CS is a self-pollinating crop with valuable agronomic attributes that makes it attractive as an alternative spring-sown crop to tight crop rotations. Seed yields of current CS varieties usually reach 2 t/ha and, the oil content of 44-46% (Vollmann et al., 2007), exceeds that of flaxseed or rapeseed. The CS oil is rich in polyunsaturated C18 fatty acids making it valuable as a renewable feedstock for oleochemical industry. Due to its high oil content and relatively stable yield potential, even under conditions of abiotic stress or reduced agronomical and chemical input, interest in CS is growing in areas where spring rapeseed yields are threatened by water or nutrient stress. Thus, several scenarios are now being considered for CS as an energy crop for advanced biofuel production. In the Plains of the United States, growth of CS on either marginal lands or as a rotation crop on fallow land can avoid conflict with food cultivation (Shonnard et al., 2010). Three years of experimentation, in the frame of CNR-Lombardia region project, gave indication that this ancient and underutilized oilseed plant can be interesting and profitable even in North Italy (Mapelli et al., 2012).

Many experiences on crops for biofuel purposes have demonstrated that the sustainability based on both the input and output energy used and on economical basis will be profitable only if the remaining flours, after oil extraction can be valorized and utilized. The evaluation of CS meal as a potential feed source is a critical factor both for sustainable biodiesel production and for increased economical value of this specie.

Moreover, the objective of this PhD thesis was the biochemical analysis of CS by-product and the study of its use in animal feed formulations. It was analyzed a collection of some varieties of CS to identify genotypes with highest protein content and lowest anti-nutritional compound levels, which negatively influence the nutritional value of this by-product.

As showed by ANOVA analysis, the results obtained indicate that CS meal protein content was significantly different both for sowing season and year of cultivation. In spring

sowing, seeds presented higher protein content respect to autumn sowing due to better nitrogen absorption in the warming season. Ligena was the genotype with highest protein content. SDS-PAGE of meal protein showed two major bands 27 and 15 kDa that probably correspond to cruciferin and napin of rapeseed. The study of amino acids (AA) profile of CS protein revealed the presence of all AA, including the ten essentials. Among the essential AA, leucine and valine were predominant. Lysine was the limiting AA in CS meal. Anyway, protein quality was quite good, in comparison with other plants generally used in animal feed like soybean or cereals. Consequently CS proteins can be considered good with balanced and appropriate AA profile. In order to evaluate the use of CS meal in animal feed diets, it was estimated the digestibility of CS meal, in ruminants, by studies “*in vitro*” of ruminal protein degradation rates and RUP (ruminal undegraded protein) analysis. The differences of digestibility of protein among the genotypes, can be explained by fibre and NDIFP (protein bound to NDF fraction) content. Fibre content influences negatively digestibility of protein especially fibre components localized in the seed coat, as reported by Matthaus (2000). Breeding of CS varieties with thinner teguments could result in a reduction of total fibre content and improve the digestibility of meal. In addition, Marles and Gruber (2004) report that the reduction of seed coat contribution in yellow seeded oilseed rape results in an increased overall contribution of the embryo to the seed weight with an enhanced oil and protein contents.

The results show that CS meal presents RUP and protein biological value similar to rapeseed (a specie close to CS) or other species generally used in animal feed formulations (ex. soybean). Thus, CS meal has the potential to be used as a protein sources in ruminants rations. *In vivo* studies are needed to further compare CS with other protein sources commonly used in dairy cattle rations.

Generally the presence of anti-nutritive compounds can limit the use of oilseed by-products in diets. The results show that the levels of sinapine, phytic acid and condensed tannins in CS meal are not dangerous for animals, and are lower respect to other Brassicaceae species, generally used in animal feed formulations. Instead the limiting factor for the use of CS flour in animal feed, both in Europe and in USA, is the content of GSLs. So it is desirable that genotypes of CS low in GSLs are selected as it was the case in the 1970-80Ss for rapeseed. The low GSL trait was first identified in the Polish spring rape variety “Bronowski”, which was then used for breeding “00-quality canola”(0 erucic acid, 0 GSLs) cultivars. Breeding program for CS to reduce GSL content is desirable to use of

CS meal in feeding of both ruminants and monogastric livestock. For this, it was necessary to develop a fast and reliable method to evaluate genotypic variation in GSL content to breed CS plants low in GSLs. A breeding for low GSL content is difficult due to the complex and interrelated biosynthesis of the different GSL compounds. The different GSL pattern observed in this thesis between CS genotypes might help breeding for low GSLs. Moreover, another approach to reduce GSL content in CS could be the relationship between GSL metabolism and amino acid composition of seed storage protein as described in *Arabidopsis thaliana* by Field et al., (2004) and in rapeseed by Malabat et al., (2003). Malabat et al.,(2003) found that meals derived from 00-quality canola seeds show a higher cruciferin/napin ratio than meals from traditional rapeseed, presumably due to an altered AA composition. It was suggested that this was due to a reduced amount of aliphatic amino acids precursor of GSLs (Malabat et al., 2003). As it shown in this thesis, GSLs and sinapine contents are inversely correlated, so it would be worth to ascertain whether a decrease in GSL content would lead to an increase in the content of sinapine.

American Feed Industry evaluated CS meal as ingredient in animal feed formulations and its potential to replace soybean meal (SBM, main ingredient used in animal feed). In Montana, the principal industry is beef cattle production. CS meal was evaluated in comparison to SBM for feed finishing beef cattle. Steers were fed 4 and 9 percent (DM basis) of a high concentrate diet of either SBM or CS meal. There were no statistical differences in the performance or weight gain of cattle fed SBM or CS diets. Sensory evaluation of steaks did not reveal any detrimental effect on taste or consumer acceptability (McVay and Lamb, 2008). The U.S Egg and Poultry Association provided supplemental funding to analyze CS meal as an ingredient for production of omega-3-rich eggs or broiler meat. CS cake seems to be a suitable feed ingredient for laying hens and broiler. CS inclusion adds protein and sulphur amino acids to feed and can replace, at least partly soybean meal.

For that, the State Department of Agriculture, American Feed Industries and the US Food and Drug Administration's (FDA) Division of Animal Feeds are working to generate documentation of what is needed to acquire GRAS (generally recognized as safe) status and feed certification for CS meal (Mc Vay and Lamb, 2008).

In this PhD thesis it was investigated about the possible use in animal feed of this new oilseed by-product after chemical extraction of oil. Feed industry needs of new rich-protein ingredient to increase animal performance, body weight and to improve the nutritional

value of meat, eggs etc. As fully described above, CS by-product, despite the GSL content that could influence negatively its use in animal feed formulations, has a good protein content, balanced amino acid profile and a quite good ruminal digestibility of protein similar to rapeseed or soybean meal generally used in animal feed.

Low-input new by-product biodiesel, CS, could strongly substitute high-input by-product biodiesel rapeseed as ingredient in animal feed formulations, as it occurred in U.S., where the introduction of CS meal in animal feed formulations was successful. The characterization of CS meal fully described in this thesis suggests that an experimentation on the use of CS by-product in animal feed in Europe could be now undertaken.

Future biochemical approach to complete the valorization of CS byproduct is the characterization of the its seed storage proteins, in particular from a nutritional point of view: it could be interesting to isolate and characterize CS napin fraction (15 kDa) and to evaluate its potential bioactivities as feed/food allergenicity, antifungal activity, calmodulin binding activity and trypsin inhibitor activity as reported for napin rapeseed by Abeysekara and Wanasundara (2009).

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