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UNIVERSIDADE DO PORTO

Sara Cristina Queirós Magalhães Sá Codeço

PROMOTING GRAIN LEGUME SEEDS IN ANIMAL FEEDING: UNVEILING THE NUTRITIVE VALUE AND PHYTOCHEMICALS OF EUROPEAN VARIETIES

Tese de Candidatura ao grau de Doutor em Ciência Animal – Ramo da Nutrição, submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

Orientador:

Professor Doutor António José Mira da Fonseca

Professor Associado

Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Coorientador:

Professor Doutora Ana Rita Jordão Bentes Cabrita

Professor Auxiliar

Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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“Remember to look up at the stars and not down at your feet. Try to make sense of what you see and wonder about what makes the universe exist. Be curious. And however difficult life may seem, there is always something you can do and succeed at. It matters that you don’t just give up.”

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List of Publications

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Paper 1

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S.C.Q. Magalhães performed seeds' phenolic compounds extraction and wrote the Introduction and Conclusions sections as well as part of the Results and discussion section.

Paper 3

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Note 1

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Communications in panel in scientific meetings

Poster 1

Magalhães, S.C.Q., Cabrita, A.R.J., Fonseca, A.J.M. Grain legumes production under rainfed conditions in Portugal. Animal Science Doctoral Programme – I Workshop, 2014, Porto, Portugal. (Annex 1)

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Communication 2

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Communication 3

Magalhães, S.C.Q., Fernandes, F., Andrade, P.B., Valentão, P., Cabrita, A.R.J., Fonseca, A.J.M. Perfil em alcaloides de variedades mediterrânicas de *Lupinus albus*, *L. angustifolius* e *L. luteus*. XIX Congresso de Zootecnia, 2015, Ponte de Lima, Viana do Castelo, Portugal. (Annex 6)

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List of Abbreviations

A	Alentejo
ADC	Apparent digestibility coefficient
ADF	Acid detergent fibre
ADL	Acid detergent lignin
amu	Atomic mass unit
aNDFom	Neutral detergent fibre assayed with heat stable amylase and expressed exclusive of residual ash
ANOVA	Analysis of variance
BI	Beira Interior
CAP	Common Agricultural Policy
CF	Compound feedstuffs
CHD	Chickpea type Desi
CHK	Chickpea type Kabuli
CNV	Catálogo Nacional de Variedades
CP	Crude protein
CV	Chickling vetch
CoV	Common vetch
DE	Digestible energy
DM	Dry matter
E&R	Estremadura e Ribatejo
EE	Ether extract
EU	European Union
FA	Fatty acids
FB	Faba bean
FM	Fishmeal
FP	Field pea
GC-FID	Gas chromatography with flame ionization detection
GC-IT/MS	Gas chromatography-ion trap mass spectrometry
GE	Gross energy
GL	Grain legumes
GM	Genetically-modified
HPLC-DAD	High performance liquid chromatography coupled to diode array detection

HPLC-DAD-ESI/MS^n	High performance liquid chromatography coupled to photodiode array detection and electrospray ionization/Ion trap mass spectrometry
IC_{25}	25%-inhibitory-concentration
LOD	Limit of detection
LOQ	Limit of quantification
5-LOX	5-Lipoxygenase
N	Nitrogen
NLL	Narrow-leafed lupin
•NO	Nitric oxide radical
NSP	Non-starch polysccharides
ODAP	3-(-*N*-oxalyl)-$_L$-2,3-diamino propionic acid
OM	Organic matter
OMD	Organic matter digestibility
P	Phosphorus
PC	Principal component
PCA	Principal Component Analysis
QA	Quinolizidine alkaloids
REF	Reference
Rt	Retention time
SBM	Soybean meal
TM	Trás-os-Montes
US	United States
UV	Ultraviolet
WL	White lupin
YL	Yellow lupin

List of International units

cm	Centimetre
g	Gram
h	Hour
ha	Hectare
kg	Kilogram
kJ	Kilojoule
L	Litre
m/z	Mass-to-charge ratio
mg	Milligram
min	minutes
mio.	Millions
MJ	Megajoule
mL	Mililitre
mm	Millimetre
mM	Milimolar
nm	Nanometre
rpm	Rotations per minute
s	Seconds
t	Tonne
U	Unit
v/v	Volume/volume
µL	Microliter
µA	Microampere
µm	Micrometre
%	Percentage
€	Euro
ºC	Celsius degrees
λ	Wavelenght

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Resumo

A União Europeia (UE) apresenta, de alguns anos a esta parte, um défice em proteína vegetal de aproximadamente 70%. Um do setores mais afetados por esta situação é a indústria dos alimentos compostos para animais que se desenvolve anualmente à custa da importação, da Argentina e Brasil, de mais de 20 milhões de toneladas de bagaços de oleaginosas, em particular bagaço de soja (BS). O BS é a fonte proteica de excelência em alimentos compostos para suínos, aves, bovinos e também peixes (em substituição da farinha de peixe) pela riqueza em proteína de elevada digestibilidade e pela composição em aminoácidos. Uma vez que a dependência externa da UE por fontes proteicas vegetais torna a produção animal vulnerável à volatilidade dos preços e a distorções comerciais, influenciando negativamente a balança comercial dos países Europeus, é agora prioridade da Comissão Europeia reverter a situação, estimulando a produção local de culturas ricas em proteína, dando enfâse às leguminosas-grão (LG). As LG são cultivadas pelos seus grãos, ricos em proteína, colhidos à maturidade e comercializados como produtos secos para consumo humano e animal. Apesar de apresentarem boa adaptação às condições edafoclimáticas da UE, a área destinada a estas culturas é atualmente reduzida (1.2 milhões de hectares em 2014), muito devido aos baixos rendimentos, sendo a sua produção (2.7 milhões de toneladas em 2014) maioritariamente destinada à alimentação animal. Um dos grandes desafios que a UE enfrenta ao incentivar a produção local de LG tem que ver com a falta de investigação, investimento e formação relativamente a práticas culturais e agronómicas, melhoramento genético e valor nutritivo destas sementes por forma a valorizá-las na alimentação não só animal mas também humana.

Neste sentido, este trabalho teve como objetivo inicial passar em revisão o estado da arte sobre a produção de LG em Portugal, uma vez que este país, a par de outros Europeus, também se apoia em elevadas quantidades de BS como fonte proteica em alimentos compostos para animais. Adicionalmente, por forma a aumentar o conhecimento sobre o valor nutritivo e composição fitoquímica de variedades Europeias de LG, foram recolhidas 51 variedades de semente com origem em diferentes países Europeus. Estas variedades, listadas no catálogo Europeu de variedades e, por isso, facilmente comercializáveis entre diferentes países, incluíram sementes de grão-de-bico (*Cicer arietinum*, do tipo Desi e Kabuli), ervilha forrageira (*Pisum sativum*), faveta (*Vicia faba* var. minor), tremoço branco e de folhas estreitas e tremocilha (*Lupinus albus*, *L. angustifolius*, e *L. luteus*, respetivamente), ervilhaca vulgar (*Vicia sativa*) e chícharo (*Lathyrus cicera*), que foram posteriormente analisadas para a composição química e perfil em ácidos gordos, carotenoides, ácidos orgânicos, compostos fenólicos e alcaloides (apenas em tremoços) usando métodos de

rotina e as técnicas de cromatografia mais indicadas. Por fim, a potencialidade de incluir variedades de LG Portuguesas em dietas de duas importantes espécies de aquacultura, nomeadamente truta arco-irís (*Oncorhynchus mykiss*) e tilápia do Nilo (*Oreochromis niloticus*), foi avaliada através de um estudo de digestibilidade usando o método de substituição da dieta e empregando o sistema de Choubert para a coleta de fezes.

Estudos nacionais apontam para a existência de diversas variedades de LG capazes de se desenvolverem sob condições de sequeiro em Portugal (sementeira de Outono) com rendimentos razoáveis (2-4 t/ha em grão-de-bico, 2-6 t/ha em ervilha e 4 t/ha em favetas) e maior peso de semente e altura da planta do que na estação de regadio (sementeira de Primavera), permitindo a colheita mecânica do grão. Trabalhos Portugueses reportando ao uso de LG como ingredientes proteicos em alimentos compostos para animais sugerem estas sementes como válidas substitutas de BS e farinha de peixe.

Relativamente às variedades Europeias estudadas, o teor médio em proteína bruta variou entre os 22 e 40 g/100 g matéria seca (MS) em sementes tanto de chícharo como do grão-de-bico do tipo Desi e tremocilha, respetivamente. O teor médio em amido variou entre 27 a 40 g/100 g MS em grão-de-bico do tipo Desi e ervilhaca vulgar, respetivamente. Não foi detetado amido nas variedades de tremoço que apresentaram, ao invés, teores mais elevados de componentes da parede celular do que as outras espécies de LG estudadas. Com a exceção das variedades de tremoço branco, para as quais o ácido oleico (C18:1*c*9) predominou entre os ácidos gordos detetados (em média 51 g/100 g ácidos gordos totais), todas as restantes variedades apresentaram o ácido linoleico (C18:2*n*6) como ácido gordo maioritário (em média 42-54 g/100 g ácidos gordos totais). Todas as variedades parecem ser boas fontes de ácido cítrico, em especial o tremoço branco (em média 385 mg/100 g MS). O grão-de-bico do tipo Desi sobressaiu relativamente às outras variedades pelo teor mais elevado de carotenoides na semente, em particular zeaxantina. Em relação ao perfil em compostos fenólicos, foram conseguidos neste trabalho importantes avanços para as LG. De facto, o perfil fenólico de sementes maduras e inteiras foi aqui caracterizado pela primeira vez para sementes de grão-de-bico do tipo Desi, ervilha forrageira e ervilhaca vulgar através de cromatografia líquida de alta eficiência com detetor de arranjo de díodos. Como sementes do género *Lathyrus* não dispunham até à data de uma caracterização detalhada do perfil fenólico, determinou-se neste trabalho o perfil qualitativo de uma variedade Portuguesa de chícharo através de cromatografia líquida de alta eficiência com detetor de díodos e espectrometria de massa com ionização por electrospray, tendo-se revelado a presença de 37 flavonoides glicosilados, a maioria do tipo kamferol. Por outro lado, para variedades de grão-de-bico do tipo Kabuli, favetas e tremoços, resultados mais aprofundados relativos ao perfil fenólico foram conseguidos para todos os genótipos em estudo. Em relação aos alcaloides de tremoço, foi também possível estabelecer pela

primeira vez o seu perfil para algumas variedades. Tendo conhecimento prévio de que extratos de tremoço ricos em alcaloides apresentam elevada atividade biológica com interesse farmacológico, determinou-se aqui, pela primeira vez, a atividade anti-inflamatória e antioxidante de extratos ricos em alcaloides de algumas variedades de tremoço, perspetivando-se atribuir maior valor e interesse a estas sementes. Os resultados mostraram que os extratos de tremoço em estudo apresentam moderada atividade anti-inflamatória, explicada parcialmente pela composição em alcaloides, mas baixa atividade antioxidante.

Finalmente, este trabalho aponta para o interesse de variedades Portuguesas de LG em dietas de truta arco-íris e tilápia do Nilo no que respeita à sua digestibilidade, havendo a necessidade de processamento prévio das sementes (como forma de aumentar a digestibilidade geral) em poucas situações. Sementes da espécie *Lathyrus cicera* (chícharo) foram aqui estudadas pela primeira vez em dietas para peixes, surgindo como ingredientes promissores.

De uma forma geral, os resultados apresentados nesta tese contribuem para aumentar o conhecimento sobre o perfil em nutrientes e em alguns fitoquímicos de variedades Europeias de LG. Adicionalmente, como primeira revisão sobre o estado da arte da produção de LG em Portugal, este trabalho poderá ser útil para os produtores nacionais e entidades com papel ativo nesta área no objetivo final de aumentar a produção de proteína vegetal e diminuir a dependência externa em BS. Finalmente, este trabalho poderá contribuir para o crescente interesse em LG no setor da aquacultura, em particular de variedades Portuguesas. Os resultados obtidos nos diferentes trabalhos contribuem, assim, para a valorização das LG para uso animal e também humano.

Abstract

The European Union (EU) shows, for several years now, a deficit in vegetable protein sources of approximately 70%. One of the sectors most affected by this situation is the compound feed industry which is annually developed at the expense of more than 20 million tonnes (mio. t) of imported oilseed meals, in particular soybean meal (SBM), from Argentina and Brazil. Soybean meal is the protein source of excellence in compound feedstuffs for swine, poultry, cattle and fish (in replacement of fishmeal) because of the high protein content (also highly digestible) and of the amino acids profile. As the EU's external dependence on plant protein sources turns animal production systems vulnerable to price volatility and trade distortions, negatively impacting the trade balance of European countries, it is now a priority of the European Commission to reverse the situation by stimulating the local production of protein-rich crops, with emphasis on grain legumes (GL). Grain legumes are grown for their rich-protein grains which are harvested at maturity and marketed as dry products for human and animal consumption. Despite well adapted to the EU's edaphoclimatic conditions, the area devoted to GL crops is currently very low (1.2 mio. ha in 2014), largely due to low yields, their production (2.7 mio. t in 2014) being mostly targeted to the animal feeding. One of the major challenges the EU faces while encouraging GL local production relates to the lack of research, investment and training on cultural and agronomic practices, breeding and nutritive value of these seeds in order to value them for feed and also food purposes.

In this context, this work aimed firstly at reviewing the state of the art on the production of GL in Portugal, since this country, along with other European countries, also relies on high amounts of SBM in compound feedstuffs. In addition, to improve the knowledge on the nutritive value and phytochemical composition of European varieties of GL, 51 seed varieties from different European countries were collected. These varieties, listed in the European Plant Variety Database, and therefore easily marketable between different countries, included chickpea (Cicer arietinum, Desi and Kabuli types), field pea (Pisum sativum), faba bean (Vicia faba var. minor), white, narrow-leafed and yellow lupins (Lupinus albus, L. angustifolius, and L. luteus, respectively), common vetch (Vicia sativa) and chickling vetch (Lathyrus cicera), and were analyzed for proximate composition and profiles in fatty acids, carotenoids, organic acids, phenolic compounds and alkaloids (in the case of lupins), using routine methods and the most advisable chromatographic techniques. Finally, the potential of including Portuguese GL varieties in the diet of two important aquaculture species, namely rainbow trout (Oncorhynchus mykiss) and Nile tilapia (Oreochromis niloticus), was evaluated

in a digestibility experiment using the diet replacement method and Choubert system for faeces collection.

National studies pointed out for the existence of several GL varieties capable of growing under rainfed conditions in Portugal (Autumn sowing) with reasonable yields (2-4 t/ha in chickpeas, 2-6 t/ha in field peas and 4 t/ha in faba beans) and higher seed weight and plant height than in the irrigation season (Spring sowing), allowing the mechanical harvesting of the grain. Portuguese studies reporting the use of GL as protein ingredients in feedstuffs suggest these seeds as valid replacers for SBM and fishmeal.

Regarding the European varieties studied, the crude protein content varied, in average, between 22 and 40 g/100 g dry matter (DM) in both chickling vetch and chickpea type Desi and yellow lupin, respectively. The starch content ranged from 27 to 40 g/100 g DM in chickpea type Desi and common vetch, respectively. No starch was detected in lupin varieties, which showed, in turn, higher levels of cell-wall components than the other GL species studied. Except for white lupin varieties, for which oleic acid (C18:1c9) predominated among the fatty acids detected (in average 51 g/100 g total fatty acids), all the other varieties showed linoleic acid (C18:2n6) as the major fatty acid (in average 42-54 g/100 g total fatty acids). All varieties appear to be good sources of citric acid, especially white lupin (in average 385 mg/100 g DM). Chickpea type Desi stood out in relation to the other GL varieties in terms of carotenoids content, in particular zeaxanthin. In relation to the phenolic compounds profile, important advances were herein achieved for GL. In fact, the phenolic profile of mature whole seeds was characterized for the first time for chickpea type Desi, field pea and common vetch by high-performance liquid chromatography coupled to diode array detection. As seeds of the genus Lathyrus were until date not characterized in detail for phenolic compounds, the qualitative profile of a Portuguese variety of chickling vetch was determined by high-performance liquid chromatography coupled to photodiode-array detection and electrospray ionization/ion trap mass spectrometry, revealing the presence of 37 glycosylated flavonoids, mainly kaempferol glycosides. On the other hand, for the varieties of chickpea type Kabuli, faba bean and lupin, a more in-depth phenolic profile characterization was obtained for all genotypes under study. In relation to lupins' alkaloids, it was also possible to establish for the first time their profile for some varieties. Knowing that lupin rich-alkaloid extracts present high biological activity with pharmacological interest, the anti-inflammatory and antioxidant activity of rich-alkaloid extracts of some lupin varieties was herein determined for the first time, with the aim of assigning more value on these seeds. The results showed that the rich-alkaloid lupin extracts present moderate anti-inflammatory activity, partially explained by the alkaloid composition, but low antioxidant activity.

Finally, this work points out to the potential of including Portuguese GL in the diets of rainbow trout and Nile tilapia, at least in what respects to their digestibility. Previous seed processing (as a way to increase overall digestibility) only seems necessary in few situations. Seeds of the species Lathyrus cicera (chickling vetch) were herein studied for the first time in farmed fish diets appearing to be promising ingredients.

In general, the results presented in this thesis contribute to increase the knowledge on the nutritive value and phytochemical composition of European varieties of GL. In addition, as a first review on the state of the art on GL production in Portugal, this work may be useful for national producers and entities with an active role in this area aiming to increase the production of vegetable protein and reduce the external dependence on SBM. Finally, this work may contribute to the growing interest in GL for the aquaculture industry, particularly of Portuguese varieties. Overall results obtained in the different works contribute, therefore, to the valorization of GL for animal and human purposes.

CHAPTER 1: GENERAL INTRODUCTION

1.1. BACKGROUND

1.1.1. Europe Union's dependence on imported protein-rich feedstuffs

1.1.1.1. Compound feed industry

Animal products are of major importance for the protein supply to the Europe Union (EU) citizens [Figure 1; 1]. In 50 years (1961-2011), the EU annual production of bovine, pig and poultry meat increased, in a total, from ca. 18 to 44 million tons (mio. t), particularly pig and poultry meat production (Figure 1). Milk production also increased in this period from ca. 121 to 156 mio. t/year [2]. No different from any other terrestrial farming activity, aquaculture production also grew in the last decades. According to the Food and Agriculture Organization [FAO; 3], aquaculture in Europe accounts nowadays for ca. 18% of its total fish production whereas in 1985 it accounted for 10%. In 2014, approximately 3 mio. t of fish were produced from coastal, marine and inland aquaculture for human consumption [3]. Particularly in the EU, the production of fish from aquaculture is of about 1.3 mio.t live weight equivalent/year, expected to increase 9% by 2025 [3].

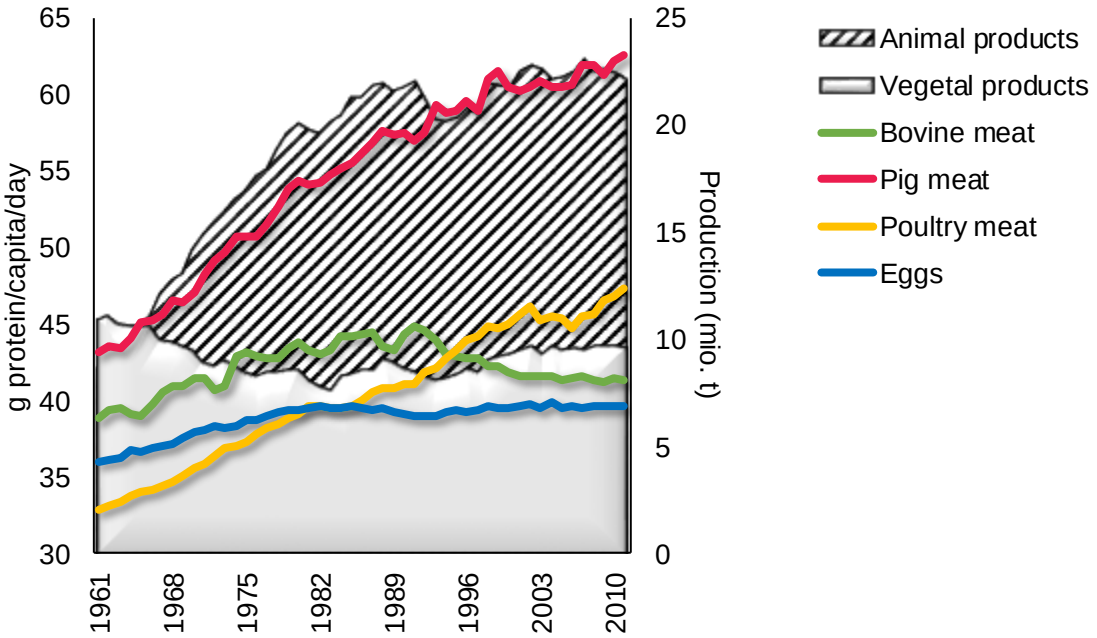

Figure 1. **Evolution (1961-2011) of daily supply of protein from animal and vegetal products and of eggs and bovine, pig and poultry meat production in the EU [1, 2].**

The growing global demand for animal proteins is expected to continue [4], driven by human population growth likely to achieve 9 billion people by 2050. The EU is self-sufficient on animal-derived products [5] but the challenge for the animal production industry is to

benefit from this human population growth while remaining competitive and sustainable on the global market [4].

Feedstuffs are the main input in animal production systems, therefore representing the most important production cost factor. Within the EU, about 480 mio. t of feedstuffs are consumed by animals each year [6]. Cattle (dairy and beef cattle and buffaloes) make use of the largest share (> 50%) while poultry, small ruminants (sheep and goats) and swine use, each, approximately 10% of the annually consumed feedstuffs [7]. Among feedstuffs consumed, ca. 49% correspond to roughages grown and used on the farm of origin, ca. 11% are cereals grown and used on the farm of origin and the remaining include feed purchased by producers to supplement their own feed resources, either feed materials (ca. 10%) or industrial compound feedstuffs [CF, ca. 30%; 6]. While cattle are responsible for most of the grass and annual forages consumption, swine and poultry make the greatest use of cereals in their diets [53% and 21%, respectively, vs. 14% in the case of dairy cows; 7].

Compound feedstuffs are in fact crucial when it comes to intensive animal production systems as they are a balanced source of essential nutrients required for body growth, maintenance, production and reproduction. The evolution of CF production in the EU and consumption by different animal species is shown in Figure 2. In 2016, the industrial CF production was of 155 mio. t, ca. 15% of the world production, of which 35% were consumed by poultry (broilers and layers), 32% by swine (piglets, pigs for fattening and breeding pigs) and 27% by cattle [fattening, dairy cows and calves; 6]. The aquaculture industry makes use of a small share of overall CF production [< 6%; 6].

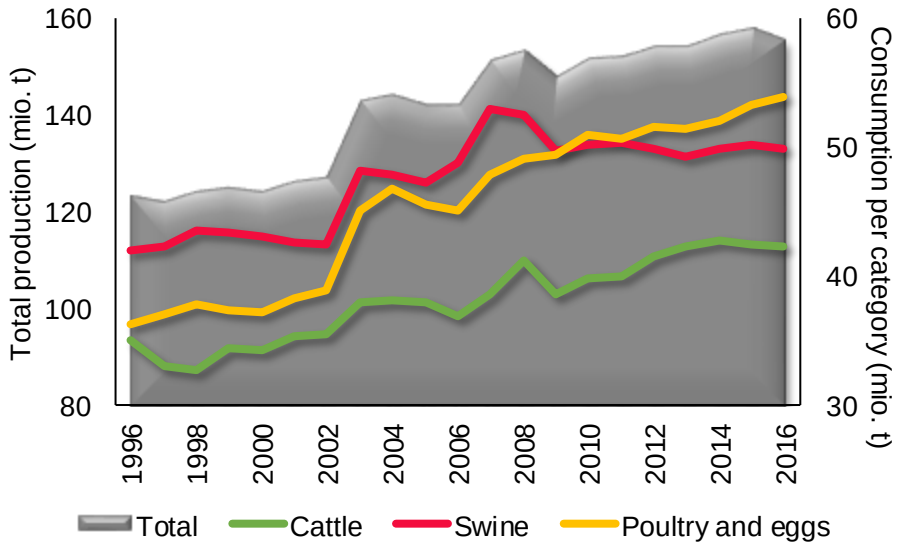

Figure 2. **Evolution (1996-2016) of compound feedstuffs production and consumption per animal category in the EU [6, 8].**

The CF consumed in the EU comprise a wide range of feed materials [9, 10], the most representative ones being cereals and oilseed meals which actually (2016) constitute 50 and 27%, respectively, of total CF raw materials (Figure 3). Tapioca, grain legumes (GL; so-called pulses), animal meals or dried forage have, in turn, decreased in expression over the years (Figure 3).

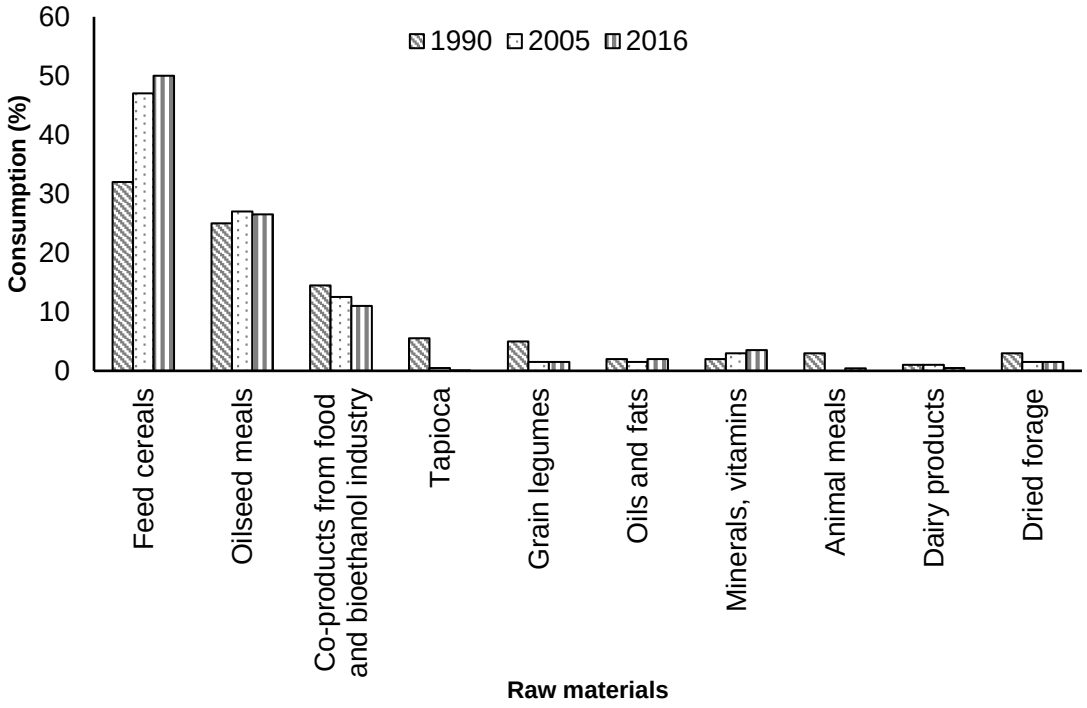

Figure 3. **Consumption of raw materials in compound feedstuffs in the EU in 1990, 2005 and 2016 [6, 10].**

The fact is that nearly 30% of the total raw materials annually consumed in the EU are imported, corresponding to more than 40 mio. t/year [Table 1; 9]. Oilseed meals are responsible for the largest share of annual imports (ca. 60%) in the industry of CF, followed by cereals (20-30%). Among the oilseed meals imported, soybean meal (SBM) leads the ranking with about 20 mio. t/year supplied mostly by Argentina and Brazil [11]. Additionally, the EU imports from Brazil and the United States (US) around 13 mio. t of soybean seeds [*Glycine max* L.; 11], a very high percentage (ca. 90%) being crushed to provide soybean oil and meal [12]. These import values associated to soybeans and SBM result from the low self-sufficiency of this protein-rich feed material in the EU, which is of only 2% [Table 2; 9].

As observed in Table 2, the overall self-sufficiency of the EU regarding protein-rich feed materials is as low as 30%, which means that the EU supplies only 30% of the protein consumed as animal feed.

Table 1. EU imports (mio. t) of raw materials for animal compound feedstuffs [9].

Raw materials	**2008**	**2011**	**2014**
Oilseed meals	27.3	26.1	23.8
Feed cereals	10.0	8.4	11.5
Molasses	2.7	1.9	1.8
Corn gluten feed	0.2	1.0	0.7
Dried distillers grains with solubles	0.2	0.7	0.6
Dried beet pulp	0.4	0.7	0.7
Citrus pulp	1.3	0.7	0.5
Fishmeal	0.5	0.4	0.3
Grain legumes	0.1	0.3	0.2
Tapioca	1.3	0.0	0.0
Miscellaneous	1.3	1.6	1.9
Total imports	45.4	41.7	43.0
Compound feedstuffs consumption	153.3	151.9	154.2
Feed import/feed consumption (%)	29.6	27.5	27.9

Table 2. EU self-sufficiency (%) on protein-rich raw materials in 2012 [9].

Protein-rich feed materials	**Self-sufficiency**
Dried forage	106
Grain legumes	94
Rapeseed and sunflower seed / meal	74
Fishmeal	67
Miscellaneous	56
Soybeans / soybean meal	2
Total	31

The EU protein deficit is not something new. It has been fluctuating between 80 and 70% in the last years [Figure 4; 9]. The import of large quantities of soybeans and by-products and the dominance of cereals in the European arable systems (section *1.1.2*) has enabled the EU to be self-sufficient in animal-derived products [13].

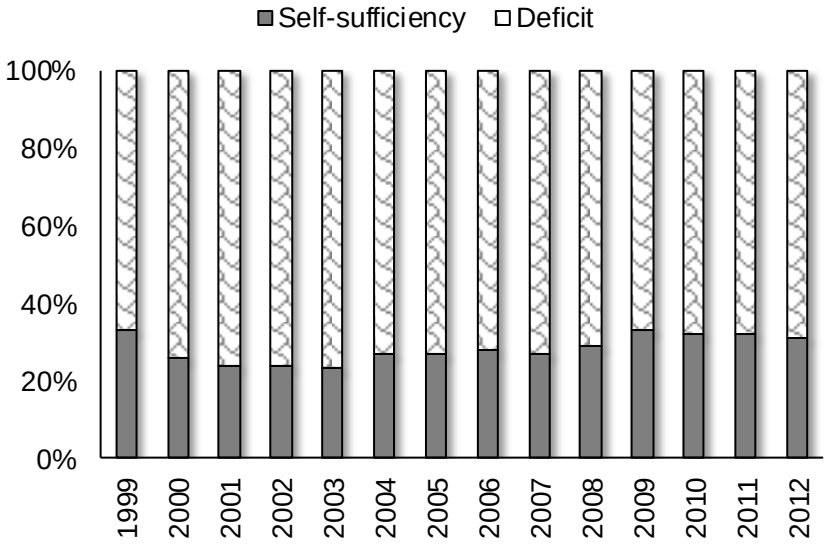

Figure 4. **Evolution of EU's self-sufficiency (and deficit) in protein sources [9].**

1.1.1.2. Soybean meal – the vegetable protein source of excellence

The statistical importance of SBM results from its value to the animal feed industry. In fact, this is considered an excellent source of protein to supplement animals' diets. Known by its high and consistent quality, SBM, to which other animal and vegetable protein sources are often compared [14, 15], consists of a highly palatable feedstuff, with crude protein (CP) levels above 400 g/kg dry matter [DM; 16, 17]. The proximate composition and the amino acid profile of SBM 44 (conventionally used in feed formulations) are presented in Table 3.

Table 3. Proximate composition and amino acid profile of SBM 44.

Proximate composition [17]	g/kg	Amino acid profile [18]	g/kg CP
Ash	62	Arginine	73.8
Crude protein	440	Histidine	27.7
Ether extract	19	Isoleucine	45.6
Crude fibre	41	Leucine	78.1
Neutral detergent fibre	91	Lysine	62.8
Acid detergent fibre	54	Methionine	14.5
Acid detergent lignin	3	Cysteine	15.2
Total sugars	70	Phenilalanine	52.6
Starch	5	Threonine	39.8
		Tryptophan	12.7
		Valine	46.9
		Total essential amino acids	454.3

Soybean meal is indeed a rich source of CP but provides low contents of starch, fibre and fat (Table 3). Its protein is highly digestible [84, 85, 87 and 90% in rabbit, swine, poultry and ruminants, respectively; 17] and well balanced in terms of amino acids (Table 3) being therefore a good complement to the amino acids present in cereals such as maize *[Zea mays* L.; 16, 19]. Typical of legume seeds, amino acids in deficit in SBM are methionine and cysteine [Table 3; 16, 17].

Considering the presence of antinutritional factors, they are lowered when soybean seeds are processed to obtain SBM, in particular protease inhibitors (4-8 g/kg CP in SBM) and the antigenic proteins glycinin (40-70 g/kg in SBM) and β-conglycin [10-40 g/kg in SBM; 20].

Soybean meal is used relatively more in some types of CF than in others. van Gelder *et al.* [21] estimated that 41, 32, 13 and 10% of the SBM processed in the EU are used in diets for swine, broilers, cattle and layers, respectively, underlining the larger dependency of monogastrics on SBM comparing to ruminants. In aquafeeds, SBM also plays an important role as this is the most commonly used vegetable ingredient to replace fishmeal given its high CP content and low levels of carbohydrates, ideal for carnivorous fish [22].

1.1.2. Protein crops in the European Union

1.1.2.1. Evolution of production and harvested area

Protein crops belong to the *Fabaceae* family and include, beyond forage legumes like clovers (*Trifolium* spp). and alfalfa (*Medicago sativa* L.), GL and soybeans [13]. Grain legumes, cultivated primarily for their rich-protein grains, are harvested at maturity, traded as dry products and consumed by humans and animals [23].

The evolution of the harvested area, production and yield of different protein crops [soybeans, lupins (*Lupinus* spp.), common bean (*Phaseolus vulgaris* L.), broad beans (*Vicia faba* L.), field peas (*Pisum sativum* L.), chickpeas (*Cicer arietinum* L.), lentils (*Lens lens* L.) and vetches (*Vicia* spp. or *Lathyrus* spp.)] in a 40-year period (1974-2014) in the EU is presented in Figure 5. In 1974, protein crops occupied an area of 3.3 mio. ha (Figure 5a) – of which more than half was intended for common bean for human consumption – producing about 2.5 mio. t (Figure 5b). In the 1980s, the areas devoted to soybean and field pea started increasing, having resulted in a combined average production of 6.3 mio. t/year that lasted until the year of 2000, being, however, nowadays of about 2.5 mio. t (Figure 5b). The other GL crops, namely, broad beans, lupins, chickpeas, lentils and vetches, have always occupied a small area in the EU (≤ 1.0 mio. ha when combined; Figure 5a); as there were

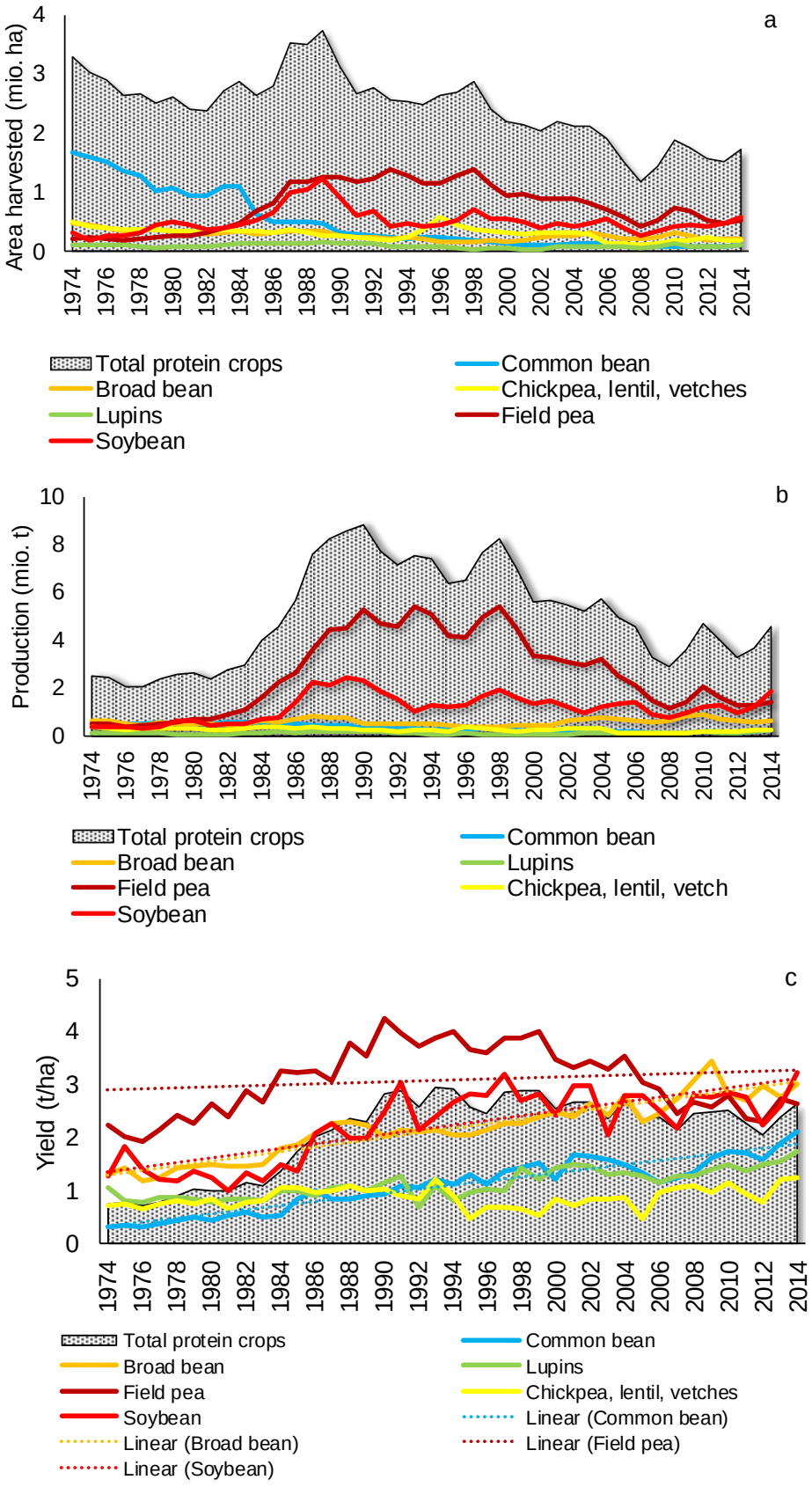

Figure 5. **Evolution (1974-2014) of the area harvested (a), production (b) and yield (c) of protein crops in the EU [2].**

no yield improvements (Figure 5c), the combined production of these GL remains, for 40 years, of only 1.1 mio. t/year (Figure 5b).

Overall, in a 40-year period, the harvested area of protein crops decreased by half (3.3 vs. 1.7 mio ha; Figure 5a), meaning that these crops are now (2014) occupying only about 2.3% of EU's arable land, which is of 72 mio. ha [12]. On the other hand, protein crops production almost doubled in this same period (2.5 vs. 4.5 mio. t; Figure 5b) meaning that yield improvements were achieved. In fact, yields increased mainly for common beans, broad beans and soybeans which are now (2014) of 2.1, 3.0 and 3.2 t/ha, respectively (Figure 5c).

Different from protein crops, cereals dominate the EU annual cropping. In fact, of the EU's arable land, cereals occupy about 58 mio. ha, this is, 80% of it [2]. Cereals production has been increasing over the years, driven by wheat's (*Triticum aestivum* L.) production increase. Together with wheat, maize and barley (*Hordeum vulgare* L.) are the most cultivated cereals in the EU [12].

1.1.2.2. Causes for the vegetable protein deficit

The drivers behind the EU protein deficit are based on several economic and policy factors and are a reflex of imbalances in the European agricultural and food systems.

Trade agreements between the EU and the US, such as the General Agreement on Tariffs and Trade in 1947 and the Blair House Agreement in 1992, are reported to be on the base of EU's protein deficit. They allowed the EU to protect its cereal production and to duty-free import protein crops and oilseeds [24, 25].

The Common Agricultural Policy (CAP) also did not encourage from the beginning the production of protein crops in the EU. In fact, the support for legumes began only in the 1970s, after the US placing an embargo on soybean exports due to production shortages and overall high global commodity prices [13]. This situation stimulated a price support for soybean, pea, lupins and faba beans and area payments for other GL in 1989, therefore resulting in increased harvested areas for such crops, especially field pea and soybean (as observed in Figure 5a). In 1992, within the MacSharry Reform, the price support was replaced by area-related direct payments and caused payments for pea, faba bean and lupins to be higher than for soybean thus decreasing soybean areas (Figure 5a). However, in 2000 (Agenda 2000 Reform), the basic amount paid per ton of protein crops was reduced [26]. In 2003, the CAP reform introduced the "decoupling system", replacing direct payments by an EU wide uniform single payment scheme not linked to production. This reform focused on cross-compliance conditions for the beneficiaries and called for more sustainable practices in the context of rural development by the member states [26]. Nonetheless,

"decoupling" end up contributing to the decline of protein crops production. In 2005, a protein crop premium was attributed for pea, faba bean and lupins, though completely decoupled in 2012 and integrated into the single payment scheme. The aim was to turn the sector more market oriented.

The lower production of protein crops comparing to cereals over the last decades in the EU is also largely attributable to the comparative yield advantage of cereals over protein crops [13, 25], making these latter less protected from international competition [4, 27]. Since protein crops are rich in protein, and in some species also in oil, they are attractive to diseases and insects, negatively impacting crops' yields. The maintenance of high quality seed stocks thus become more difficult and leads to a comparatively short storage life of seeds [28]. For example, the yield of wheat [5.6 t/ha; 2] is about twice that of protein crops (Figure 5c). The variability of GL's yields across Europe was recently reviewed by Cernay *et al.* [25]. With cereals and protein crops competing for the same land, arable crop farmers base their decision on the economic output they can get from each crop, logically preferring to grow cereals in the farm rotations [4]. Grown as cash crops with immediate income, cereals expanded during decades in production and yield mainly due to available low-cost nitrogen (N) fertilizers, investment in plant breeding and in a wide range of pesticides [13, 28].

The decline in the direct human consumption of GL is another reason given for the reduction in the area of protein crops [27]. For example, GL crops used exclusively for human consumption, namely common bean, chickpea, lentil and broad bean, occupied the EU cropping in 1974 with 2.7 mio. ha; currently (2014), the area devoted to those GL is approximately 5-fold smaller [Figure 5a; 2]. According to Bues *et al.* [13], GL in humans diets have been replaced by meat. Apparently, the area of protein crops in some Mediterranean countries declined less than in other EU regions because of the prominent role of legumes in the regional diet [13].

Training and acquisition of practical experience in domestic protein crop production were also neglected over the years, leading to a low level of innovation on protein crops seed production in the EU [24]. Meanwhile, significant progresses were accomplished outside the EU on the efficiency of protein crop production and on the use of new technologies, leading to a competitive disadvantage for EU farmers who take protein crops production as economically unattractive.

Finally, the ban on the feeding of meat and bone meals to cattle, sheep and goats in 2001 further worsened the EU protein dependency by 4% [4]. The EU restricted the use of processed animal meals in CF in 2001 due to the arise of bovine spongiform encephalopathy in ruminants [29]. Animal meals, which comprised nearly 7% of CF in the 1990s, experienced a sharp drop in its rate of incorporation in the 2000s; this is evident from Figure 3. Meat and

bone meals present an average CP content of 550-600 g/kg DM [30] and therefore the highest protein levels among major feed ingredients. For example, field peas, faba beans and lupins present a CP content of about 210, 260 and 350 g/kg DM, respectively. Field pea was widely used in the past combined with meat and bone meals to obtain a product with an average CP content high enough to be attractive for swine and poultry feed [26]. However, the exclusion of meat and bone meals from CF also reduced the appeal for field peas; the subsequent decline in GL use in CF is also evident from Figure 3.

1.1.2.3. Consequences of the protein deficit

The low self-sufficiency on protein crops exposes the EU to possible dubious trade practices and to scarcity and price volatility of soybean on the global market. The price of SBM has been irregularly increasing over the years, recently achieving historical prices, for example 472€/t in august 2012 [31]. The price of non-genetically modified (GM) SBM is even higher [32]. Cost and availability of SBM are strongly correlated with the price of agricultural commodities, which is, in turn, influenced by population and economic growth, changes in the consumer's product preferences and weather conditions [15]. In addition, any problem in one of the main soybean producer countries, this is, US, Argentina and Brazil, will have immediate consequences on the global market and especially on the SBM prices [4]. Moreover, with China for five years now as the main soybean buyer, the EU faces a reduced control over soybeans supply and also an insecure position regarding the unpredictability of soybean prices in the global market [27].

The use of GM plants as food or feed is also a particularly controversial issue. In fact, GM varieties are widely adopted in the main soybean export countries with more than 90% of the globally traded soybeans estimated to be GM. Only less than 15% of the ca. 30 mio. t of soybeans and derived products annually imported to the EU are identity-preserved certified GM-free [33]. No detrimental effects of GM compared to non-GM feedstuffs appear to exist [34] and there is large market acceptance of GM crops in animal feed. However, there is still a small sector of the market requiring certified GM-free feeds [33]. Noting, the EU has a very stringent regulatory framework for GM crop import for food and feed use [35] which may lead to an asynchronous approval between the EU and non-EU countries and induce soybean trade disruptions [36].

From another perspective, social, economic and environmental consequences derive from the lack of protein crops in the EU cropping systems. In what respects to the EU, the specialization and intensification of cereals production during the second half of the 20th century was highly dependent on external inputs (pesticides, fertilizers) and mechanization,

leading to low proportion of permanent grasslands in the landscape and overall simplified crop rotations [37]. Mainly in western Europe, mixed livestock and arable farms reduced in number while farm size increased [37]. Farming systems became quite homogenous [13], with further impacts on agricultural ecosystems and on their sustainability [37]. Indeed, this arable management impacted plant and animal communities, soil characteristics and water and air quality [37, 38] with widespread decline of farmland biodiversity, necessary to the ecological requirements of many species [13, 37, 39]. This agricultural intensification predominated in the north. In eastern Europe, the extensive systems rapidly moved towards intensification and abandonment in the 1990s leading to the emigration of rural people and loss of traditional farm buildings [37].

1.1.3. European Commission alert on the protein deficit

With the protein deficit in the EU being a long-standing problem (Figure 4), the EU Parliament set up a motion in 2011 [24] that called for putting more effort in breeding, research and development to increase the EU's own production of protein-rich materials. This topic deserves to be assessed with accuracy and thus considered a relevant objective. Therefore, some opportunities and challenges to the production of protein crops in the EU are described below.

1.1.3.1. Opportunities for protein crops production

A dedicated policy is an essential element to stimulate the European production of protein crops. Since 2013, promoting protein crops has become a priority of the CAP, with the focus being on pea, faba and broad beans, chickpea, lupin and soybean [27]. Measures under the new CAP reform for the period 2014-2020 that most stimulate the production of protein crops relate to greening measures and voluntary direct supports [40]. Concerning to greening measures, producers shall dedicate 5% of their land to areas of ecological interest, with legumes, as N-fixing crops, being highly valued in this regard, and cultivate two different cultures in farms with more than 10 ha and three crops in farms with more than 30 ha, to promote crop diversification [13]. Under the new CAP, and in line with the EU 2020 Strategy, a group of the Agriculture European Innovation Partnership is only dedicated to protein crops, aiming to investigate their potential in the EU crop rotations and to make suggestions on how to increase their productivity and seeds protein content [4, 41].

Increasing the cultivation of protein crops would be an important contribution to the sustainable development of EU agriculture and food systems [27]. Indeed, crops from the

Fabaceae family, while able to fix N from the air and to store it in root nodules, reduce the need for synthetic fertilizers, further decreasing N losses, pollutant emissions and the use of fossil energy [27, 42, 43]. These crops are also effective in recovering unavailable forms of soil phosphorus (P), an expensive and often limiting resource in many cropping systems [27, 28]. The plant residues, rich in N, when left on the soil help to reduce the need of the next crops for fertilizers. On the other hand, the presence of protein crops in cropping systems, for instance in rotation with cereals, increases biodiversity, improves soil fertility and lowers the incidence of weeds, diseases and pests [27, 42].

The increased prices also of synthetic fertilizers constitute another opportunity for protein crops' production in the EU. As abovementioned, legume crops can replace this major input in agriculture becoming more attractive for farmers and occupying a more competitive position than before [13].

Another opportunity to produce protein crops in the EU relates to the increased demand by the population on more information about the background of food products. Indeed, people are concerned about products quality, their social/historical aspects and the ethics with which they are produced, processed and traded. This means that consumers' awareness in relation to health, social responsibility and authenticity is increasing. If on the one hand most of the SBM imported is from GM cultivars and a GM-free supply chain for EU's animal feed industry would come closer to the cultural values of its citizens, on the other, there is increasing interest on local food systems [32]. In addition, organic farming systems do not accept GM ingredients and oilseed products subjected to solvent extraction processes [15].

During the past two decades, considerable research on protein crops has been developed in Europe [41]. Examples of projects in execution in 2017 on this topic are Eurolegume and LEGATO (on legumes; 2014-2017) and PEAMUST (on pea; 2012-2019) [41]. Given the importance GL is reaching, the 68th United Nations General Assembly declared 2016 as the International Year of Pulses [44] aiming to develop worldwide the consumption of GL through increased publicity, promotion of health benefits and product innovation.

1.1.3.2. Challenges to the production of protein crops

If the EU is willing to increase its area and production on protein crops, the major challenge to be faced relates to the improvement of their productivity/yields [32]. According to Roman *et al.* [27], soybean and GL (field peas and faba beans) should increase their yields in 30 and 69-76%, respectively, in order to be competitive with wheat, and in 63 and 112-

120%, respectively, in order to be competitive with maize. To achieve this goal, technical innovations on breeding and agronomy are needed. Breeding of protein crops is mostly restricted to the public or semipublic sectors because of the relatively small market for these crops in the EU at the moment, one of the challenges being the involvement also of the private breeding industry [32]. Also necessary for the implementation of these crops in the EU is the agronomic research on cultivation and rotational aspects. In fact, it is important to know more about variety choice, fertilization, disease control, water use, crop mixtures and environmental benefits [32].

The EU farmers risk aversion for growing legume species has to decrease [25]. It urges to train farmers about protein crops: their agronomic features, benefits when in rotation with other crops and savings on the use of chemical inputs such as fertilizers and pesticides. It is crucial to provide them information on the most advantageous varieties in terms of yield and productivity, as well as, in relation to mechanical harvesting facility (whenever this is feasible).

1.1.3.3. Focus on grain legumes and thesis layout

As mentioned above, the emphasis on the European production of protein crops to overcome the shortage in vegetable protein relies on soybeans and GL. Comparing to GL, soybeans present some disadvantages, as follows. As a tropical crop, soybeans require specific climatic conditions to growth, namely four months of warm and rainy conditions to reach maturity [23]. Additionally, most soybeans, even those produced in the EU, are GM. This constitute a major limitation for soybeans because the EU legislation on GM ingredients is very rigid and also because the public opinion, i.e. consumers, are increasingly opting for more organic food products not including GM's in their diets. Moreover, soybeans always require the processing of seeds oil extraction to obtain the meal. In turn, GL cultivated in Europe (Figure 6) are Mediterranean crops easily adapted to most of EU's edaphoclimatic conditions. Besides that, they are non-GM and may be used without processing, depending mainly on the level of antinutritional factors. In Europe, animal feeding is the principal outlet for GL [19].

Figure 6. **Examples of grain legumes produced in the EU.**

Getting advantage of this, the interest on GL for animal feeding has increased and this is easily observed through recent publications on the topic reviewing the potential of these ingredients in animal nutrition [15, 19, 45-47], namely of pigs, farmed fish or cattle. However, as previously shown in Figure 3, this recent interest in GL is not yet reflected in an increased use of these ingredients in animal CF.

In Portugal, the animal feed industry is one of the most important sectors in the national agri-food context following meat and dairy industries [48]. Along with other EU countries, it also relies on impressive amounts of oilseed meals in animal CF, particularly SBM [almost 0.5 mio. t in 2015; 49]. In 2015, Portugal imported ca. 0.8 mio. t of soybean seeds from Brazil (a part being further processed into meal) and 0.1 mio. t of SBM from the US [48]. In contrast, only approximately 1000 t of GL were used in national CF, namely, field pea, faba bean and sweet lupin, this latter re-introduced in 2015 after 11 years of total absence in feedstuffs [48]. As, according to Häusling [24], several GL crops are adapted to the European climatic conditions, for example faba beans, field peas, lentils, lupins, chickpeas, **Chapter 2** of the present dissertation, entitled "*Grain legumes production under rainfed Portuguese conditions for animal feeding: A review* ", includes a manuscript, to be improved for publication, on the state of the art of GL production in Portugal and on their use in animal feedstuffs.

If locally-grown GL are intended to increase in animal CF as protein-rich ingredients, more research is needed on the chemical profile of available marketable varieties. It is important to improve knowledge on seeds not only for proximate composition but also for the presence of secondary metabolites, in that benefits or drawbacks may arise thereof. In this sense, a further insight on the chemical characterization of particular European varieties of GL (from Portugal, Spain, France, Italy and Poland) was envisaged, focusing on seeds nutrients and non-nutrients. Therefore, **Chapter 3** of the present dissertation, entitled "*Proximate and phytochemical composition of European varieties of grain legumes*", presents the chemical characterization of European varieties of GL regarding proximate composition and profiles on fatty acids, carotenoids and organic acids.

One of the most relevant classes of phytochemicals in crops in general respect to phenolic compounds. Besides contributing to growth, reproduction and defense of plants and to the seeds sensory characteristics, they display several biological activities, their main one being the antioxidant activity, to which most health benefits have been attributed to [50]. This makes it essential to study the phenolic profile of GL seeds and, in this sense, some work has already been done for some species on immature grains considered vegetables for human consumption [e.g.; 51, 52, 53]. However, regarding raw seeds harvested as mature and whose main target is the animal feed industry little data is available. **Chapter 4** of the present dissertation includes, therefore, an already published paper in Food Chemistry journal entitled "*European marketable grain legumes seeds: Further insight into phenolic compounds profiles*" which reports the qualitative and quantitative profiles in phenolic compounds of several varieties of GL seeds. Among GL, it was noticed that chickling vetch seeds (*Lathyrus cicera* L.) had never been studied before for their phenolics composition, despite their interest in food and feed, high crop resilience and already existing low neurotoxin 3-(-*N*-oxalyl)-L-2,3-diamino propionic acid lines [54]. Hence, **Chapter 5**, includes an already published paper in Food Chemistry journal entitled "*HPLC-DAD-ESI/MS^n profiling of phenolic compounds from Lathyrus cicera L. seeds*" reporting the qualitative phenolics' profile a Portuguese variety of chickling vetch.

Within different species of GL, lupin seeds present the advantage of containing greater CP values (ca. 30-40 g/100 g DM) being therefore highly valued when it comes to replace high protein ingredients such as SBM or fishmeal in animals' diets [e.g.; 14, 55]. Nonetheless, the main limitation of lupin seeds, in what concerns to secondary metabolites, are the alkaloids, mainly the quinolizidine alkaloids [56]. When ingested by humans, acute toxicity of these metabolites can cause neurological, cardiovascular and gastrointestinal disturbances; in feedstuffs, while conferring a bitter taste to the diet, alkaloids may decrease its palatability, decreasing feed intake and affecting animals' body weight gain [56]. If on the one hand, alkaloids may be toxic when ingested at high concentrations, on the other, several

biological properties were already described for rich-alkaloid lupin extracts and which end up contributing to the valorization of this crop for other purposes rather than for food or feed. In this regard, **Chapter 6** of the present dissertation includes a published paper in Industrial Crops and Products journal entitled "*Alkaloids in the valorization of European Lupinus spp. seeds crop*" reporting the qualitative and quantitative profile in alkaloids of several European lupin seed varieties as well as the pharmacological potential of lupin rich-alkaloid extracts by the determination of their antioxidant and anti-inflammatory activities.

Production of aquatic animals from aquaculture has shown in the last decades an impressive growth in the supply of fish for human consumption being considered the world's fastest growing food production sector [3]. Fishmeal and fish oil are still considered the most nutritious and digestible ingredients for aquafeeds, however, their incorporation in CF has been showing a clear downward trend given the high historical prices these raw materials are achieving and the increasing awareness on more sustainable practices along the food chain [3]. Instead, fishmeal and fish oil are now being selected as strategic ingredients for specific stages of production (hatchery, broodstock and finishing diets) and used at lower dietary concentrations. Following this, efforts have been made by industry and academia towards finding alternatives to fishmeal and fish oil in aquafeeds. In this sense, GL could function as total or partial replacers of fishmeal given their low price and flexibility in providing both protein and energy to diets. **Chapter 7** of the present dissertation includes therefore a published paper in Aquaculture Nutrition journal entitled "*Apparent digestibility coefficients of European grain legumes in rainbow trout (Oncorhynchus mykiss) and Nile tilapia (Oreochromis niloticus)*" reporting the apparent digestibility of six Portuguese GL varieties in the diet of two important freshwater fish species in aquaculture.

An overall discussion of the works presented along this dissertation can be found in **Chapter 8**, entitled "*General discussion, conclusions and future perspectives*". It also includes the major conclusions of the studies carried out as well as some future perspectives regarding the use of grain legumes in animal feeding.

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1.2. AIMS OF THE STUDY

In line with the actual promotion of GL production in Europe as a way of decreasing the animal feed industry external dependence on protein-rich raw materials such as soybeans and SBM, the general objectives of this dissertation were to understand the specific situation of Portugal regarding protein crops production for animal feedstuffs, to unveil the nutritive value and phytochemical profiles of European marketable varieties of GL and, finally, to evaluate the potential of GL in the diet of important fish species for the aquaculture industry. As so, the specific purposed aims were as follows:

- To review the state of the art on GL production in Portugal both from agricultural and animal feeding points of view;
- To gather, from European seed companies, varieties of different GL species, namely of chickpeas (*Cicer arietinum* L.), field peas (*Pisum sativum* L.), faba beans (*Vicia faba* L. var. minor), white lupins (*Lupinus albus* L.), narrow-leafed lupins (*L. angustifolius* L.), yellow lupins (*L. luteus* L.), common vetches (*V. sativa* L.) and chickling vetches (*Lathyrus cicera* L.), focusing on seeds belonging to the European Plant Variety Database [57] given their ease of commercialization;
- To determine varieties proximate composition;
- To characterize varieties fatty acids profile;
- To establish varieties phytochemical profiles regarding phenolic compounds, carotenoids and organic acids as well as alkaloids in the case of lupins;
- To evaluate the apparent digestibility coefficients of GL varieties in the diet of rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*).

CHAPTER 2: GRAIN LEGUMES PRODUCTION UNDER RAINFED PORTUGUESE CONDITIONS FOR ANIMAL FEEDING: A REVIEW

GRAIN LEGUMES PRODUCTION UNDER RAINFED PORTUGUESE CONDITIONS FOR ANIMAL FEEDING: A REVIEW

Sara C. Q. Magalhães, Ana R. J. B. Cabrita, José M. F. Abreu, António J. M. Fonseca

REQUIMTE/LAQV, ICBAS, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal

Abstract

In a European scenario of external dependence on protein crops for the animal industry, grain legumes (GL) production in Portugal arises as an opportunity to equilibrate the country's trade balance. Chickpea (*Cicer arietinum* L.), field pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.) and lupins (*Lupinus* spp. L.) are important GL in Mediterranean farming systems, with some tradition in Portugal. The present work aimed at reviewing the state of knowledge on the Portuguese production of such GL as well as on their nutritive value for animal feeding. National studies clearly show the existence of GL varieties capable of growing under Portuguese rainfed conditions (Autumn sowing) with reasonable grain production (ca. 2000-4000 kg/ha for chickpeas, 2000-6000 kg/ha for field peas and 4000 kg/ha for faba beans) and with higher seeds weight and plant height than in the irrigated season (Spring sowing), ultimately allowing mechanical harvesting. These parameters are easily improved through irrigation two to three times at the end of the culture. Portuguese works reporting the use of GL as protein ingredients for feedstuffs suggest these seeds as valid replacers of other protein sources commonly used. However, the present study appeals for the need of more exhaustive work assessing GL chemical composition, use extent and impact on animal growth and performance.

KEYWORDS: animal feeding, legumes-based rotations, grain legumes, sowing date, vegetable protein sources

1. Introduction

The European Union is deficient in vegetable protein sources, one of the most affected sectors being the animal feed industry, as the massive production of meat, milk and/or eggs requires a large contribution of compound feedstuffs. In Portugal, of the total raw materials used in animal feedstuffs, 23% corresponds to oilseed meals with soybean meal (SBM; *Glycine max* L.) representing about 70% of that value [1]. However, soybean production in Portugal is limited due to climatic and market constraints. Rapeseed (*Brassica napus* L.) is also produced, but in slight amounts despite its recognized potential at least in the inland North [2]. Hence, one alternative to import oilseeds (or their meals) could be the national production of grain legumes (GL), as stated by the European guidelines [3].

Chickpea (*Cicer arietinum* L.) is cultivated in Portugal since ancient times [4]. Conversely, field pea (*Pisum sativum* L.) has only developed at an industrial scale in 1986, expecting to occupy a large area, which was not observed [5] mainly due to the huge competition by other crops adapted to regions of Atlantic influence. Portugal is considered one of the main faba bean (*Vicia faba* L.) producers in Europe [6]. It is grown throughout the country, with some agronomic and economic importance in the South [7]. Within lupins, yellow lupin (*Lupinus luteus* L.) is the one with the longest tradition given its tolerance to acidic and low fertile soils [8]. Its production is mainly targeted to animal feeding either as grain or forage. White (*Lupinus albus* L.) and narrow-leafed (*Lupinus angustifolius* L.) lupins are also cultivated [9]. However, as most white lupin varieties are sweet, this is, with low levels of alkaloids, they are mainly consumed by humans, being considered of greater economic importance than the other lupin species [10]. Green manure is another priority associated with the cultivation of yellow and narrow-leafed lupins [10].

Despite the suggested potential of Portugal to produce GL [11], the actual area occupied by these cultures is limited [12]. Several Portuguese authors have referred the first Common Agricultural Policy measures as responsible for the focus of agriculture production on Winter cereals, and not including legume crops in rotations [13]. The use of inadequate varieties and agronomic criteria that have led to low yields [< 1000 kg/ha per year; 12] and compromise economic return, can comprise another argument for farmers' disinterest [14].

The objective of this work was to review the production of GL in Portugal focusing on varieties of field peas, chickpeas, faba beans and lupins. These species are relevant in Mediterranean farming systems [15] and of interest, among others, to the animal feed industry and to the on-farm dietary supplementation. The eventual increase on GL production could have a substantial impact on improving the country's trade balance.

2. Grain legumes under rainfed Portuguese conditions

2.1. Edaphoclimatic conditions

Portugal is a country with Mediterranean influence, with wide precipitation variability (most of it occurring between Autumn and Spring, this is, from October to March), periodic droughts and sudden and intensive downpours. The different agricultural regions that compose mainland Portugal are presented in Figure 1 and the descriptive statistic of each weather conditions is detailed in Table 1. In almost all the west coast of mainland Portugal and in numerous mountainous regions prevail a temperate climate with dry warm Summer (Csb, according to Köppen-Geiger climate system classification), whereas in the majority of the southern central plateau regions and in the Mediterranean coastal regions prevail a temperate climate with dry hot Summer (Csa, according to the same climate system classification). In a small region of Baixo Alentejo, namely in the district of Beja, a cold steppe climate can also be observed [BSk according to the same climate system classification; 16].

Figure 1. **Agricultural regions of mainland Portugal.**

Approximately 96% of the soils directed to agricultural production present medium to low cation exchange capacity (< 20 meq/100 g soil) and 88% a pH below that considered optimal for plant growth [< 6.5; 17]. Indeed, acidic soils prevail all around the country except for some regions in the coastal centre and south. Moreover, around 70% of the soils contain low levels (ca. 1%) of organic matter [OM; 17]. Only the regions of Entre-Douro e Minho, Beira Litoral and the alluvial zones of Ribatejo (Figure 1) present soils with medium-high OM

levels, which are allocated to more intensive agricultural systems. In some regions with steep slopes, soils are highly vulnerable to erosion through precipitation.

Table 1. Descriptive statistic of the characteristics of the weather conditions of mainland Portugal's agricultural regions, for the period 1971–2000 [16].

	Minimum[1]	Maximum[2]	Mean[3]	SD
Minimum temperature in the coldest month (°C)				
Entre-Douro e Minho	0.2	5.5	3.7	0.97
Beira Litoral	0.8	6.6	3.6	0.96
Trás-os-Montes	-1.5	3.8	0.8	0.80
Beira Interior	-1.0	4.9	2.2	1.17
Estremadura e Ribatejo	2.4	9.8	4.8	1.25
Alentejo	2.8	9.2	4.8	0.64
Algarve	5.1	9.5	6.7	0.93
Maximum temperature in the hottest month (°C)				
Entre-Douro e Minho	18.5	31.8	26.1	1.92
Beira Litoral	20.5	31.6	27.0	1.83
Trás-os-Montes	18.6	33.8	28.3	2.18
Beira Interior	16.9	35.5	29.5	2.52
Estremadura e Ribatejo	19.1	32.6	28.5	2.59
Alentejo	21.7	35.9	31.6	1.93
Algarve	22.2	32.7	28.5	1.68
Precipitation in the month with the lowest monthly total precipitation (mm)				
Entre-Douro e Minho	9	69	29	8.3
Beira Litoral	5	43	15	5.6
Trás-os-Montes	5	60	17	7.2
Beira Interior	1	44	10	4.5
Estremadura e Ribatejo	1	20	7	2.1
Alentejo	1	20	4	2.1
Algarve	1	19	2	1.6
Precipitation in the month with the highest monthly total precipitation (mm)				
Entre-Douro e Minho	132	571	281	66.8
Beira Litoral	74	436	181	52.2
Trás-os-Montes	68	512	140	72.3
Beira Interior	68	447	129	50.7
Estremadura e Ribatejo	68	281	121	22.5
Alentejo	68	227	99	18.3
Algarve	68	291	137	29.1
Number of days with precipitation < 0.1mm annual[4]				
Entre-Douro e Minho	218	255	235	7.2
Beira Litoral	233	267	251	7.3
Trás-os-Montes	230	282	262	10.6
Beira Interior	239	298	270	12.6
Estremadura e Ribatejo	243	284	268	8.8
Alentejo	260	304	285	8.7
Algarve	278	310	293	9.0

Values were calculated from the grids of the Climatic Atlas of Portugal, obtained by interpolation of the mean values, for the period 1971–2000. SD, standard deviation.

[1] Location with the lowest value for the indicated climatic parameter (except for the number of days with precipitation < 0.1 mm annual). [2] Location with the highest value for the indicated climatic parameter (except for the number of days with precipitation < 0.1 mm annual). [3] Average value of the

indicated climatic parameter. [4] Year with the lowest (minimum) and highest (maximum) number of days with precipitation < 0.1 mm.

2.2. Nitrogen fixation

Grain legumes, as all legumes, have a distinctive feature that allows them to establish a symbiotic relationship with bacteria of the genus *Rhizobium*, being able to fix nitrogen (N) and to store it in root nodules. Carranca *et al.* [15] reported, in a Portuguese Haplic Luvisol, annual N fixation values, under regular rainfall, from 76 to 125 kg/ha for faba bean and from 31 to 107 kg/ha for field pea. High amounts of fixed N by faba bean were also reported on a Vertisol, in Spain [18, 19]. In South Australia, 81 kg fixed N/ha/year by field pea were recorded [20]. Regarding lupins, Carranca *et al.* [21] found, in a Haplic Podzol in Portugal, above 100 kg fixed N/ha/year by white lupin, and Castro [22], in a Cambisol, 89 kg/ha/year of fixed N by yellow lupin. Comparatively to field pea, faba bean and lupins, chickpea usually fixes less N [19]. Indeed, according to Kumar and Abbo [23], chickpea can fix up to 140 kg N/ha/year, but is more usual to find values ranging from 20 to 60 kg N/ha/year. Beyond symbiotic N fixation, lupins are also able to use insoluble forms of phosphorus from the soil [24], thus resulting agronomic, environmental and economic advantages.

2.3. Sowing season

In Mediterranean conditions, faba bean, field pea, and lupins, as rainfed crops, can be sown in Autumn/Winter [25, 26]. Conversely, chickpea was traditionally sown in Spring in Portugal and in other Mediterranean countries, as the varieties normally used present no resistance to low Winter temperatures and to a fungal disease caused by *Ascochyta rabiei* (Pass.) Lab. [27]. This infection develops in cool and wet weather [27] like occur in our Winter. However, this constraint has been solved by the selection of *Ascochyta* blight tolerant and resistant varieties in the National Institute of Agriculture and Veterinary Research (INIAV, I.P., Portugal), by combining local with exotic material [28], that allow the anticipation of the sowing date. However, the main actual constraint is the lack of sufficient amount of seeds for the development of the culture. Among chickpeas, distinction must be made between Kabuli and Desi types; the former represents Mediterranean, large, white to cream seeds, usually intended for human's diets while the latter represents Indian, small and dark seeds commonly used in animal feeding [29, 30] and in coffee manufacturing. Figure 2 presents the effects of sowing season of chickpeas and field peas (included or not in the National Catalog of Varieties [CNV; 31]; Table 2) on grain yield (kg/ha), plant habit (cm) and 100 seeds weight (g) observed in Portuguese studies. Noting, varieties included in the CNV are considered

along this work in separate from those not included in the CNV because the formers, while registered in the European Plant Variety Database [32], can be easily traded between European countries.

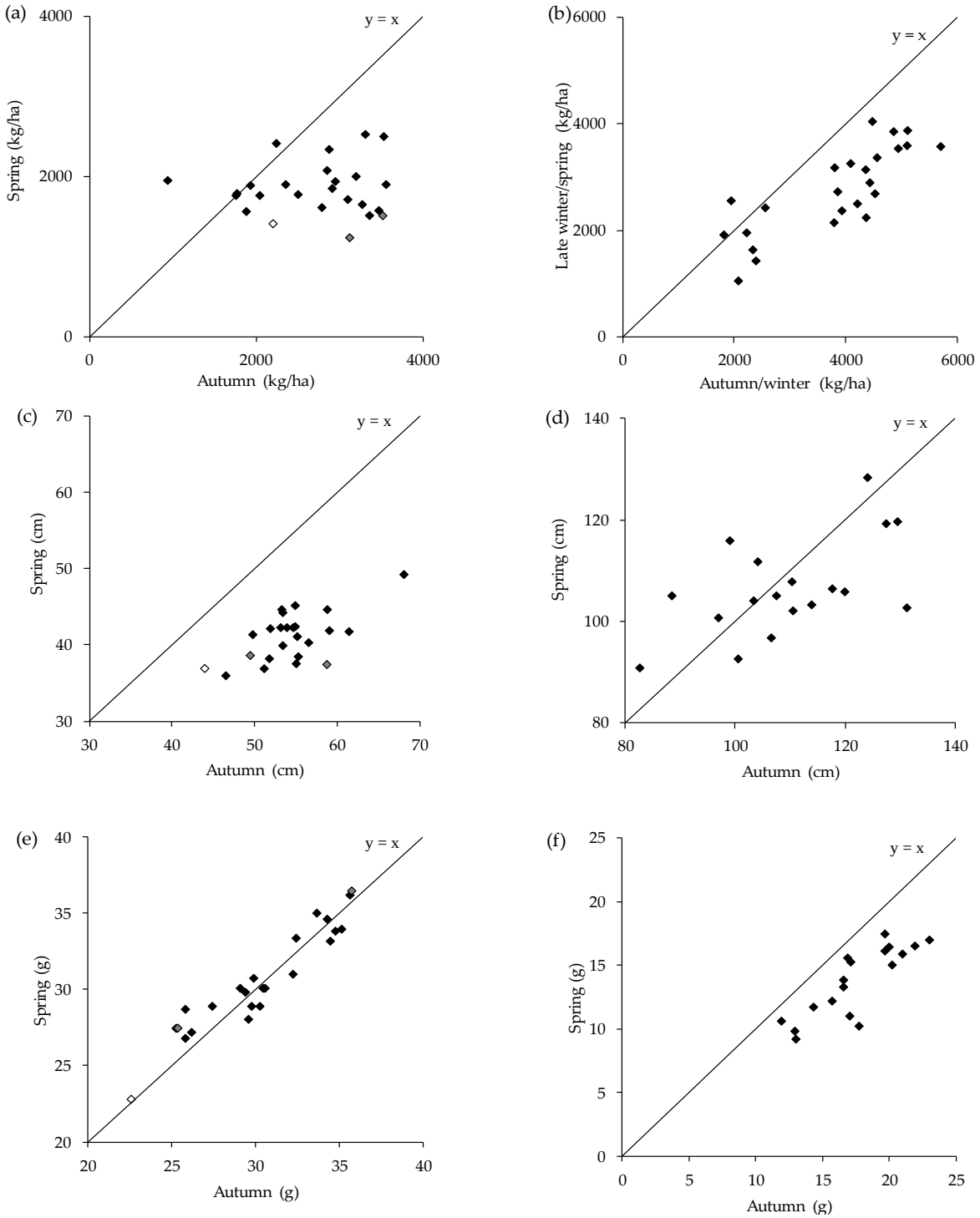

Figure 2. **Effect of sowing season on (a) grain yield (kg/ha) of chickpeas [33, 34], (b) grain yield (kg/ha) of field peas [5, 34], (c) plant habit (cm) of chickpeas [34], (d) plant habit (cm) of field peas [34], (e) 100 seeds weight of chickpeas [34] and (f) 100 seeds weight of field peas [34].** ◊,

Portuguese chickpea Kabuli type varieties [31]; ♦, Portuguese chickpea Desi type varieties [31]; ♦, other varieties (foreign and Portuguese not included in the CNV [31]).

Under these experimental conditions and comparing to Spring, Autumn sowing promotes higher chickpea grain yield, from an average of 1827 (± 307.2) kg/ha in Spring to 2698 (± 699.8) kg/ha in Autumn, representing a yield increase of 48% (Figure 2a). These results agree with those found in other Mediterranean countries. For instance, grain yield increases of 70% in Syria [35], between 23 and 188% in Greece [36] and and more than 50% in Spain [37] were reported.

The same trend was observed for field pea (Figure 2b), with an increase of 39% in grain yield (averaging 2748 ± 814.1 kg/ha in Spring and 3822 ± 1155.4 kg/ha in Autumn). Peksen et al. [38], in Turkey, reported a grain yield increase of 103%, when field pea was sown in Autumn comparing to Spring (6640 kg/ha vs 3270 kg/ha, respectively). In France, field pea Autumn sowing increased the grain production in 1000 kg/ha [39].

Forwarding sowing season to Autumn promoted chickpea plant habit (54 ± 4.9 cm vs 41 ± 3.2 cm; Figure 2c), thus allowing mechanical harvesting as plant habit heights between 55 and 65 cm were considered the most adequate for total crop mechanization [40]. Conversely, field pea plant habit did not seem to be affected by sowing season (Figure 2d). Autumn sowing promoted heavier field pea seeds (Figure 2f), seeming to have no effect on chickpea seeds weight (Figure 2e).

The effect of sowing date of faba bean (*minor* var. Pragana) on grain yield is shown in Figure 3.

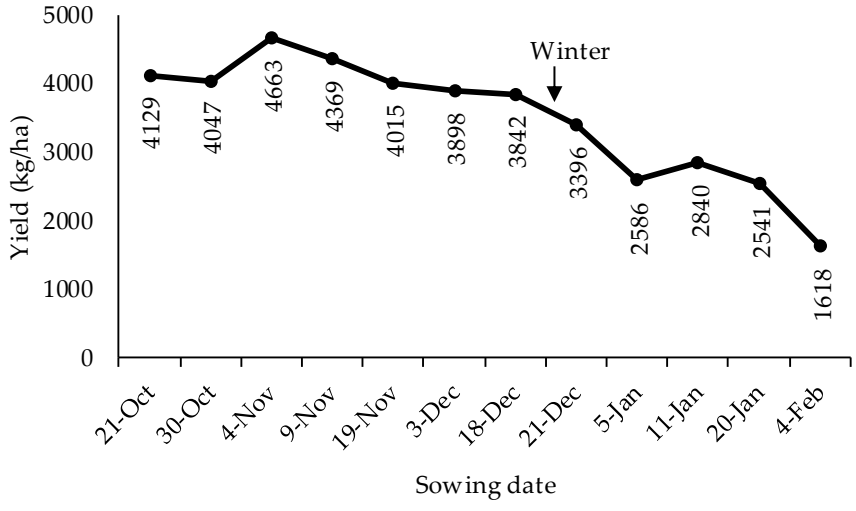

Figure 3. **Effect of sowing date on grain yield (kg/ha) of faba bean (*Vicia faba* L. minor var. Pragana) sown in Alentejo, Portugal [33].**

Autumn sowing dates promoted higher grain production than Winter ones, the highest (4633 kg/ha) and the lowest (1618 kg/ha) grain yield being observed for the sowing dates of

4th November and 4th February, respectively. Loss and Siddique [41] also reported, in dryland Mediterranean-type environments of South Western Australia, decreases in faba bean grain yield with the delay of sowing date from Autumn to Winter. Although we were unable to find Portuguese studies comparing different sowing dates on lupins production, others [42, 43] performed in Mediterranean countries (Turkey and Western Australia) have showed that sowing at the beginning of the rainy season (generally October), when the soil is still warm, increases grain yield and yield components.

The results presented in the Portuguese literature suggest clear advantages of Autumn sowing comparing to late Winter or Spring. Indeed, delaying GL sowing to late Winter or Spring brings disadvantages or constraints, namely high temperatures and sun irradiation and irregular or scarce rainfall, that lead to heat and drought stresses towards maturity, shortening of growing cycle, low and irregular yields, unsuitable plant habit and consecutively the need of manual harvesting [36].

2.4. Multi-site yield experiments

Figure 4 presents the grain yield obtained by Portuguese GL varieties [Table 2; 31] when sown in Autumn and grown under rainfed conditions in different agricultural regions in Portugal.

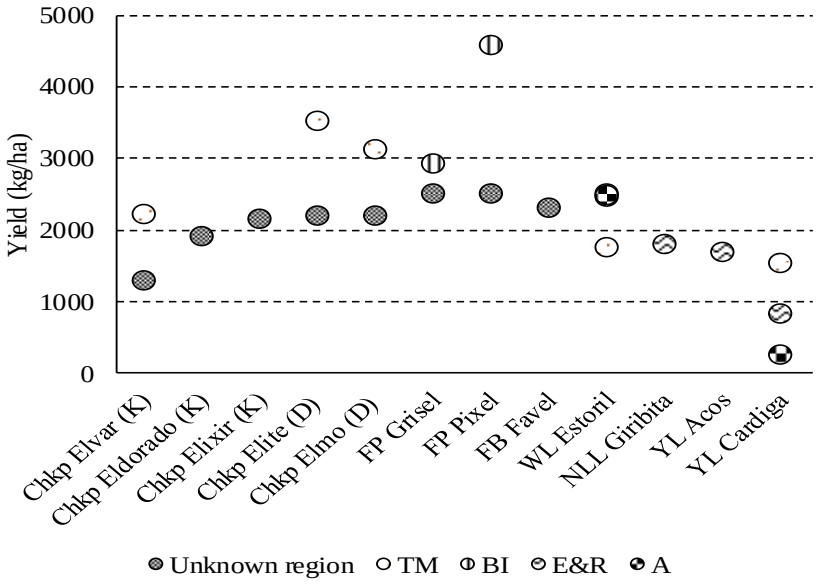

Figure 4. **Grain yield (kg/ha), in Autumn sowing of Portuguese varieties [31] of Kabuli (K) and Desi (D) types of chickpea [Chkp; 34, 44], field pea [FP; 44, 45], faba bean [FB; 44], white lupin [WL; 10, 46], narrow-leafed lupin [NLL; 46] and yellow lupin [YL; 10, 30, 46, 47]. A, Alentejo; BI, Beira Interior; E&R, Estremadura e Ribatejo; TM, Trás-os-Montes.**

Table 2. Varieties of chickpea, field pea, faba bean and lupin included in the National Catalog of Varieties [31].

Grain	Variety	Maintenance	Country
Chickpea	Elite (Desi type)	INIAV, I.P.	Portugal
	Elmo (Desi type)	INIAV, I.P.	Portugal
	Eldorado (Kabuli type)	INIAV, I.P.	Portugal
	Elixir (Kabuli type)	INIAV, I.P.	Portugal
	Elvar (Kabuli type)	INIAV, I.P.	Portugal
Field pea	Grisel	INIAV, I.P.	Portugal
	Pixel	INIAV, I.P.	Portugal
	Esmeralda	Semillas, EL Solc S.L.	Spain
	Marqueta	Semillas, EL Solc S.L.	Spain
	Montrebei	Semillas, EL Solc S.L.	Spain
	Monsant	Semillas, EL Solc S.L.	Spain
Faba bean	Favel	INIAV, I.P.	Portugal
White lupin[1]	Estoril	INIAV, I.P.	Portugal
Narrow-leafed lupin[1]	Giribita	INIAV, I.P.	Portugal
Yellow lupin[1]	Acos	INIAV, I.P.	Portugal
	Cardiga	INIAV, I.P.	Portugal

[1] All Portuguese lupin varieties are sweet (with low alkaloids levels), with the exception of yellow lupin var. Cardiga [10, 48].

The varieties of chickpea Kabuli type (Elvar, Eldorado and Elixir) yielded, in average, between 1300 and 1900 kg/ha whereas Desi type ones (Elite and Elmo) slightly surpassed the 2000 kg/ha (Figure 4). Chickpea yields were higher in Trás-os-Montes region than in the unknown region. The yield of field pea varieties was registered between 2500 and 3000 kg/ha, Pixel having almost achieved 5000 kg/ha in Beira Interior (Figure 4). The Favel variety (faba bean *minor*) grain yield was similar to those of some chickpea varieties and white lupin var. Estoril yielded between 1800 and 2500 kg/ha in all agricultural regions (Figure 4). Grain yields from 1700 to 1800 kg/ha were reported for the variety Giribita of narrow-leafed lupin and for the yellow lupin var. Acos, in the central West region (Estremadura e Ribatejo). The lower seed yield of the yellow lupin var. Cardiga (Figure 4) agrees with its genetic improvement towards green biomass production [10], thus being difficult to obtain grain given its extreme dehiscence [30].

The results of Portuguese multi-site experiments in Autumn/Winter sowing with other varieties (foreign and Portuguese ones not included in the CNV) of chickpea, field pea and sweet white, narrow-leafed and yellow lupins are summarized in Figure 5. Chickpea Kabuli type seeds (Figure 5a) showed higher average grain yield in Trás-os-Montes (2564 ± 668.9 kg/ha), followed by Estremadura e Ribatejo (2305 ± 485.6 kg/ha) and Alentejo (2076 ± 601.2

kg/ha). Fewer varieties of Desi chickpea than of Kabuli chickpea were studied, however, yield results are similar between these two chickpea types for the same regions (Figures 5a and 5b). Beira Interior is the region with the lowest yield results for Desi chickpea (Figure 5b).

Field pea grain yields above 3000 kg/ha were observed in all agricultural regions tested, the best performances being found in Beira Interior, where 50% of the varieties yielded between 3955 and 6264 kg/ha (Figure 5c).

Sweet white lupin varieties sown in Alentejo showed wide performances variation (Figure 5d). Higher average grain yields were achieved in Trás-os-Montes and Beira Interior (1986 ± 256.7 kg/ha and 2659 ± 355.8 kg/ha, respectively; Figure 5d). The low yield values observed in Alentejo for some sweet (462 ± 135.1 kg/ha; Figure 5d) and bitter [810 ± 220.6 kg/ha; 30] white lupin varieties agree with this crop higher requirements in terms of soil [49], as in this agricultural region predominate low fertile, sandy and acidic soils [10]. Sweet varieties of narrow-leafed lupin showed higher and similar average yields in Trás-os-Montes (1021 ± 203.3 kg/ha) and Estremadura e Ribatejo (1232 ± 204.4 kg/ha; Figure 5e). In Alentejo, bitter varieties of narrow-leafed lupins yielded more [958 ± 84.5 kg/ha; 30] than sweet ones (636 ± 47.6 kg/ha; Figure 5e). Miranda and Rebelo [47] also reported higher yields with increased content of alkaloids in the seed of yellow lupin. Indeed, Portugal intends large part of yellow lupin areas to bitter varieties possibly due to their lower purchase price and greater hardiness [50]. However, sweet yellow lupins show good performances in Trás-os-Montes (with an average grain yield above 2000 kg/ha; Figure 5f). It must also be noted that sweet lupins are more likely to be eaten by several predators due to their sweetness [51].

In general, the yield results presented for the Portuguese GL varieties (Figure 4) agree with those of the foreign or Portuguese varieties not included in the CNV (Figure 5). From these multi-site yield experiments in Portugal, it can be concluded that the Northeast of Portugal (Trás-os-Montes) is another interesting region for chickpea production, despite the limited actual production [52]. Indeed, Alentejo, and Estremadura e Ribatejo have been the main agricultural regions responsible for chickpea production in Portugal with 1046 and 181 t produced in 2015, respectively [52].

Similarly, despite field pea adaptation to a wide range of agronomic conditions throughout the country, Beira Interior appears to be a great region for field pea production as higher yields are obtained compared to the other regions. Finally, sweet lupins present themselves with interesting yields in several Portuguese regions.

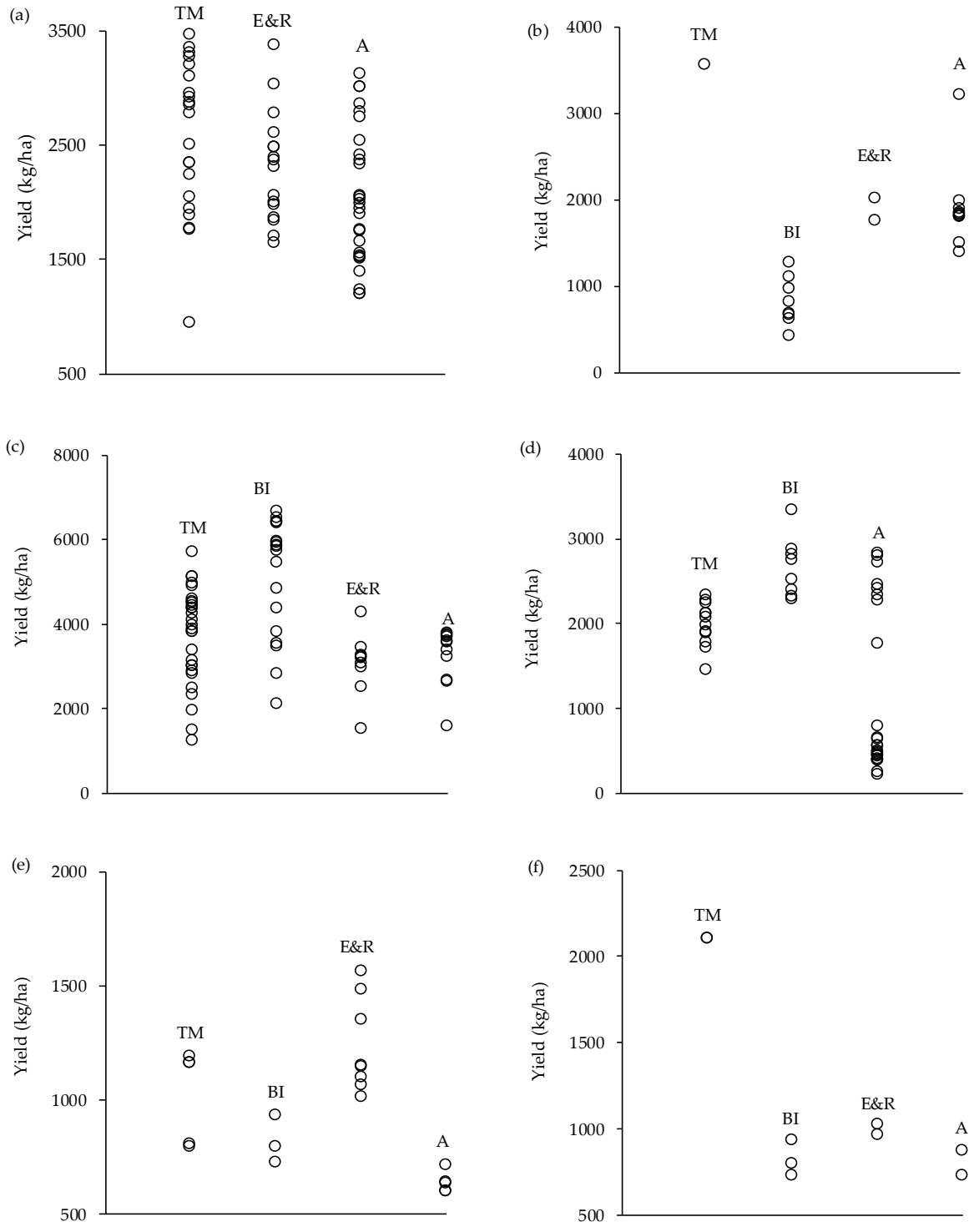

Figure 5. **Grain yield (kg/ha), in Autumn/Winter sowing, in Portugal, of foreign or Portuguese varieties not included in the CNV [31] of (a) chickpea Kabuli and (b) chickpea Desi types [34, 53-55], (c) field pea [34, 45], (d) sweet white lupin [10, 30, 47], (e) sweet narrow-leafed lupin [10, 30, 46, 56] and (f) sweet yellow lupin [10, 30, 47, 56]. A, Alentejo; BI, Beira Interior; E&R, Estremadura and Ribatejo; TM, Trás-os-Montes.**

2.5. Legume-based rotations

Actually (2015), only 4% (ca. 142154 ha) of the mainland Portugal utilized agricultural area is directed to rainfed cereals grain production, namely of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), triticale (x *Triticosecale* L.) and rye [*Secale cereale* L.; 1]. Along with the reduced production area, the grain yields of such cereals are typically reduced, contributing to the low self-sufficiency degree, observed in Portugal, mainly regarding wheat and rye [7 and 14%, respectively; 57]. Those cereals are normally sown in Autumn/Winter (early sowing), normally in N poor fields, and grown under rainfed conditions, especially in the lowlands of the North and Centre inland regions (Trás-os-Montes and Beira Interior) and in the South of the country (Alentejo). The cereal-fallow rotation was the most common system due to N and water economies, as well to the common belief that soil fertility is restored, and weeds and diseases cycles are broken through fallow [58]. However, cereal monoculture systems are not recommended, as they increase the farmer risk exposure associated with production and price variations and are also less environmentally friend [59].

It is well documented that the introduction of GL in rotation can greatly contribute to the development of the following cereal [60, 61]. Briefly, GL high N to carbon ratio and the capacity to make available other nutrients lead to an increase in the OM content of soils. Also, the persistence and incidence of pests and diseases both in cereals and GL is decreased due to the allelopathic effect of rotation [62]. With a better use of natural resources, minimized tillage practices, lower inputs dependence, and decreased greenhouse gases emissions and N leaching, GL-cereal rotations lead to more economic and environmentally friend farming practices, contributing to a more sustainable agriculture [61, 63]. Simultaneously, biodiversity is encouraged and landscapes are enhanced [59].

As far as we know, there is a lack of Portuguese work on this subject. Castro [22] evaluated, in a 10-year study, the effects of a cereal (wheat and triticale)-yellow lupin and a cereal (wheat and triticale)-fallow rotations on both cereals performance, in a Cambisol in Trás-os-Montes (Table 3). Although not statistically significant, higher yields and N contents of cereal grain and straw were found when in rotation with the GL, rather than in a cereal-fallow system (Table 3). When calculated per kg of N in the previous crop residue, Carranca *et al.* [64], in a 2-year study in a Haplic Luvisol in Estremadura e Ribatejo, reported an increase in oat biomass when preceded by white lupin comparatively to a continuous oat-oat. Salgueiro [65] already stated that lupins are interesting to include in rotation with less demanding cereals, such as oats, rye and triticale (being also able to rotate with wheat in poorer soils), while chickpeas, field peas and faba beans are more indicated for wheat- and barley-rotations, given their similar soil requirements.

Table 3. Effect of yellow lupin and fallow in a cereal (wheat and triticale)-based rotation (in Alentejo, Portugal) on cereals' grain and straw yield and nitrogen (N) content and on 1000 seeds weight [22].

Cereal	Rotation	Grain yield	Grain N	Straw yield	Straw N	1000 seeds weight
		kg/ha	g/kg	kg/ha	g/kg	g
Wheat[1]	Yellow lupin	2420	20.2	7000	5.3	40.3
	Fallow	2220	17.4	5710	3.8	44.9
Triticale[2]	Yellow lupin	2930	15.7	6510	3.6	34.2
	Fallow	2480	14.1	5990	3.4	32.5

[1] Rotation effects on wheat were evaluated during the first six years of the trial; [2] Rotation effects on triticale were evaluated during the last four years of the trial.

3. Grain legumes in animal feeding

In Portugal, the consumption of GL in animal compound feedstuffs has been inconstant over the years [66, 67]. Actually, only field peas [subspecies hortense; 68] and faba beans [minor varieties; 69] are used. In 2015, Portugal resorted on 687 t of field peas (less 61% than in 2014) and on 483 t of faba beans, the same as in 2014 [67]. Sweet lupins were used until 2004 [70] being again included in CF in 2015 [84 t; 71]. Chickpea production in Portugal is entirely directed for human consumption [1], suggesting a limited production of Desi type varieties, not attractive as food for humans except when used in soaps and as mashed chickpea [72]. In what concerns to lupins, yellow lupins are mostly used in extensive farming systems, where sheep either graze the whole dry plant during the Summer months [50]. However, the seeds produced on-farm can also be offered indoors after maceration (soaking and imbibition in water) to reduce the alkaloids content [73].

3.1. Nutritive value

There are some but not many Portuguese studies focusing on the use of GL in animal feedstuffs. Data on the nutritive value of GL produced and/or used in Portugal is somehow scarce, crude protein (CP), ether extract (EE) and starch being the most analysed chemical parameters. The published data on chemical composition and nutritive value of Portuguese and other (foreign and Portuguese not included in the CNV) varieties of chickpea, field pea, faba bean and lupins are presented as Supplementary Material (Table S1, Table S2, Table S3 and Table S4, respectively).

The highest CP and EE contents are found in lupins, namely in yellow and white ones, respectively. Indeed, CP content of yellow lupins surpasses 400 g/kg dry matter (DM) and white lupins EE content is above 100 g/kg DM. Similar values are reported by Petterson [74]. Chickpeas CP contents both for Kabuli (182-240 g/kg DM) and Desi types (201-238 g/kg DM)

are in accordance with Bampidis and Christodoulou [29]. Starch constitutes a large fraction of GL seeds, comprising around 400-500 g/kg DM, lupins being an exception, as non-starch polysaccharides constitute up to 30 to 40% of their seeds [75]. Literature reports higher starch contents for field pea [68, 76] and lower ones for chickpeas [29], relatively to the average summarized values found in the Portuguse studies (400 and 389-472 g/kg DM, respectively). Comparatively, the most used vegetable protein source in animal feeding, SBM, presents higher CP content (ca. 440 g/kg DM) than all GL, low EE levels (ca. 15 g/kg DM) like field peas and faba beans, and irrelevant starch values as lupins [76].

Reflecting GL chemical composition, higher in vitro gas production was found for chickpea [77, 78] and field pea varieties when compared to lupins given their higher starch content [78]. Similarly, Guedes and Silva [79] found for both DM and N degradation kinetics in the rumen of adult cows significant higher values for the slower degradable fraction – *b*, in the Ørskov and McDonald [80] equation – in lupins relatively to field pea. These results agree with the findings of Calabrò *et al.* [81] in which lower gas production and slower fermentation kinetics were described for lupins comparatively to field peas and faba beans. Organic matter digestibility in ruminants is similar between GL species [800-920 g/kg DM; 77, 78], and is in line with the values presented by INRA [76].

Portuguese studies reporting the amino acidic fraction of GL proteins highlight the seeds lower content in methionine and cysteine and the high contents in lysine [82-84]. Indeed, the lack of tryptophan and sulphur amino acids is one of the main constraints of GL [85]. One other is related with the presence of antinutritional factors, already described in earlier works [86, 87]. Achieving a low concentration of undesirable substances in plants is essential for human and animal nutrition and is considered a real challenge for plant breeders [88]. It should be noted that compounds categorized as antinutritional may also have beneficial properties for the health of the consumers by revealling, for instance, biological activity [48]. The chemical characterization of non-nutrient compounds in GL must, therefore, be performed in detail [48, 89, 90].

3.2. Portuguese in vivo trials

Portuguese in vivo trials with GL in animals' diets mainly report to rabbits, pigs and farmed fishes, most of them focusing on the replacement of the commonly used protein sources by legume seeds. Studies indicate for the possibility of replacing SBM in the diet of growing rabbits by up to 40% of chickpeas (Kabuli type) or 20% of faba beans without affecting their productive performance [91, 92]. It is suggested by the authors that the antinutritional factors of chickpeas Desi type and faba beans may impair higher levels of inclusion of these seeds in growing rabbits' regimens. Also, field peas were already reported

to be a satisfactory SBM substitutes when included at a level of 30% in the diets of rabbits, improving fertility and food conversion rates [93].

In weaned piglets, less favourable situations with GL-containing diets were described and comprise ileal losses of protein [94], systemic antibody responses due to the allergenicity of GL proteins [95], decrease of both protein digestibility and duodenum enzymes activity and villus atrophy [84], and potential worsening of animal performance [96]. These effects seem more pronounced with lupins due to their high content in structural carbohydrates [82, 83, 97], the supplementation with hydrolases not having improved protein ileal digestibility [98]. Conversely, Prandini *et al.* [99] did not found negative performance effects with high levels of lupins (170 g/kg) in weaned piglets and, overall, lupins, field peas and faba beans were considered satisfactory alternative protein sources to SBM [83, 99] and good complements to cereal proteins [82].

In growing pigs, lupins fibre fraction was also an issue negatively affecting energy digestibility [100]. However, both lupins and field peas are considered good vegetable protein sources for these animals also showing hypocholesterolemic properties [101, 102]. Prandini *et al.* [99] suggest an inclusion up to 100-150 g/kg of lupins and 150-200 g/kg of field peas in the diet of growing pigs. Other authors did not find major issues on growth and slaughtering performances of pigs fed GL nor with the digestive utilization of nutrients [103, 104].

Portuguese studies also clearly demonstrate the possibility of including between 30 and 66% of lupins, field peas or faba beans, as replacers of fish meal, in the diet of farmed fish such as rainbow trout [105], European sea bass [106, 107], and gilthead seabream [108], with no negative associated effects, being in accordance with other authors [109]. Recently, Magalhães *et al.* [110] showed that raw Portuguese GL varieties present potential in terms of digestibility to be included in diets for both rainbow trout and Nile tilapia with previous seed processing being apparently required only for chickpeas and faba beans in rainbow trout and for chickling vetch (*Lathyrus cicera* L.) in Nile tilapia diets.

As far as we know, there are no published Portuguese studies on the effect of GL in ruminants' performance. Nevertheless, the conclusions of several existing works are unanimous in reporting GL as readily accepted ingredients and valid substitutes of SBM in sheep [111, 112] and cattle [113], with, for instance, no constraints related to feed intake and milk yield or composition. According to Dixon and Hosking [114], the two main limitations of GL, namely the lack of sulphur amino acids and the presence of antinutritional factors, are of lesser importance for ruminants than for monogastrics due to the fermentation reactions occurring in the rumen provided that these characteristics are taken into account when formulating the diet. Thus, with few exceptions, it seems not necessary to resort on processing techniques to improve GL nutritive value for these animals, allowing the reduction

of production costs. Also, contrarily to what was above mentioned for swine, lupins high fibre content do not constitute a problem in ruminants [114].

3.3. Conclusions

Research evidence presented in this review shows that there are several chickpea, field pea, faba bean and lupin varieties, adapted to Portuguese soils and climatic conditions, capable of growing under rainfed conditions (Autumn sowing), with final grain yields above those traditionally observed. Grain legumes-cereal rotations could benefit the cereal and contribute to more organic and extensive farming systems, leading to higher farmer incomes while helping to combat human desertification in North and Central inland, and South (Alentejo) of the country.

Available data on the chemical composition and nutritive value of GL used in Portugal suggest these seeds as interesting protein sources for animal feeding. Indeed, *in vivo* studies emphasize the potential of these ingredients as alternatives to the commonly used protein sources in livestock and aquaculture industries.

Focusing on the varieties that show a good adaptation to Portuguese ecological conditions, this paper appeals for the need of more exhaustive work related with GL nutritive value, with detailed characterization of their antinutritional factors and of treatments feasible to be routinely applied to decrease or even eliminate their levels. It is also important to evaluate GL extent of use and impacts on growth and performance of animals reared in intensive farming systems (swine, poultry, fish and ruminants) and whose diets largely resort on imported protein-rich compound feedstuffs.

Supplementary material: Table S1: Chemical composition (g/kg DM) of varieties of chickpea Kabuli and Desi types (*Cicer arietinum* L.); **Table S2:** Chemical composition (g/kg DM) of varieties of field pea (*Pisum sativum* L.); **Table S3:** Chemical composition (g/kg DM) of varieties of faba bean (*Vicia faba* L.); **Table S4:** Chemical composition (g/kg DM) of varieties of lupin (*Lupinus* spp.).

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Table S1. Chemical composition (g/kg DM) of varieties of chickpea Kabuli and Desi types.

Cultivar	CP	EE	CF	NDF	ADF	ADL	Starch	Sugar
Portuguese Kabuli[1]								
Elvar [40, 44]	233	-	-	-	-	-	433	-
Eldorado [44]	226	-	-	-	-	-	-	-
Elixir [44, 110]	234	-	-	143	30	17	413	-
Mean	231	-	-	-	-	-	423	-
SD	4.2	-	-	-	-	-	10.0	-
Other Kabuli[2]								
ChK 256 [77]	182	64	30	130	48	3.1	499	64
ChK 283[3] [78]	215	52	39	109	58	13.0	460	55
ChK 309 [115]	221	56	-	-	-	-	-	-
ChK 510 [115]	216	61	-	-	-	-	-	-
ChK 512 [115]	216	62	-	-	-	-	-	-
ChK 513 [115]	214	60	-	-	-	-	-	-
ChK 551 [115]	215	60	-	-	-	-	-	-
ChK 571 [115]	215	61	-	-	-	-	-	-
ChK 606 [115]	215	61	-	-	-	-	-	-
ChK 807 [115]	215	61	-	-	-	-	-	-
ChK 881 [115]	219	62	-	-	-	-	-	-
ChK 1081 [115]	222	57	-	-	-	-	-	-
FLIP 8315C [77]	205	58	36	146	58	6.3	491	87
FLIP 82186C [77]	221	55	38	160	57	4.2	459	73
FLIP 82258C [77]	207	63	31	146	64	1.4	542	75
FLIP 8341 [77]	213	56	37	158	59	1.0	498	64
ILC 482 [77]	200	63	39	127	55	4.2	504	63
Unknown [92]	240	61	39	109	59	10.5	452	-
Unknown[4] [84]	195	43	-	101	32	0.1	345	-
Mean	213	59	36	132	54	4.9	472	69
SD	11.9	4.9	3.6	22.1	9.3	4.38	55.4	10.5
Portuguese Desi[1]								
Elmo [44, 110]	233	-	-	229	94	15	345	-
Elite [44]	238	-	-	-	-	-	-	-
Mean	235	-	-	-	-	-	-	-
SD	3.8	-	-	-	-	-	-	-
Other Desi[2]								
ChD 322 [115]	207	56	-	-	-	-	-	-
ChD 323[5] [78, 115]	203	49	91	169	121	22.0	382	-
ChD 326 [115]	215	60	-	-	-	-	-	-
ChD 1083 [115]	225	58	-	-	-	-	-	-
ChD 1085 [115]	217	51	-	-	-	-	-	-
ChD 1087 [115]	219	50	-	-	-	-	-	-
ChD 1090 [115]	210	52	-	-	-	-	-	-
ChD 1091 [115]	213	51	-	-	-	-	-	-
PCH 70 [77]	226	51	54	163	101	6.3	503	26
Unknown [92]	201	62	90	164	119	13.0	409	-
Unknown[6] [84]	213	40	-	191	102	11.3	261	-
Mean	214	53	78	172	111	13.2	389	-
SD	8.1	6.1	21.1	12.9	10.7	6.55	99.7	-

ADF, acid detergent fibre; ADL, acid detergent fibre; CF, crude fibre; CP, crude protein; DM, dry matter; EE, ether extract; NDF, neutral detergent fibre.

[1] Included in the CNV [31]; [2] Foreign or Portuguese varieties not included in the CNV; [3] 921 g organic

matter digestibility (OMD)/kg DM; 17.6 MJ digestible energy (DE)/kg DM (evaluated in Merino rams); [4] 337.9 g non starch polysaccharides (NSP)/kg; 8.3 g ash/kg; [5] 902 g OMD/kg DM; 16.9 MJ DE/kg DM (evaluated in Merino rams); [6] 414.1 g NSP/kg; 28.5 g ash/kg.

Table S2. Chemical composition (g/kg DM) of varieties of field pea.

Cultivar	Ash	CP	EE	NDF	Starch
Portuguese[1]					
Grisel [44]	-	215	-	-	-
Pixel[3] [44, 110]	-	223	-	197	432
Mean	-	219	-	-	-
SD	-	5.8	-	-	-
Others[2]					
Gp 950[4] [78]	33	210	17	146	453
Cartouche [116]	35	231	11	-	412
Enduro [116]	34	228	11	-	407
Audit [116]	34	242	14	-	450
Corrent [116]	36	261	8	-	389
Alhambra [116]	35	250	15	-	397
Cherokee [116]	36	245	16	-	424
Isard [116]	35	249	12	-	405
Livia [116]	37	239	7	-	368
Gregor [116]	35	250	9	-	408
James [116]	35	232	11	-	417
4740[5] [79]	40	301	23	264	362
Unknown[6] [105]	19	246	-	-	-
Unknown[7] [83]	-	199	17	135	311
Mean	34	242	13	182	400
SD	4.9	23.8	4.4	71.7	37.6

CP, crude protein; DM, dry matter; EE, ether extract; NDF, neutral detergent fibre.

[1] Included in the CNV [31]; [2] Foreign or Portuguese varieties not included in the CNV; [3] 34.5 g lipids/kg DM; 66.3 g acid detergent fibre (ADF)/kg DM; 271.3 g non-starch carbohydrates/kg DM; [4] 72 g crude fibre/kg DM; 81 g ADF/kg DM; 11 g acid detergent lignin (ADL)/kg DM; 911 g organic matter digestibility/kg DM; 16.5 MJ digestible energy/kg DM (evaluated in Merino rams); [5] 804 g degradable DM/kg DM (with a rumen outflow rate of 4.4%/h and a degradation rate of 0.131/h); 878 g degradable nitrogen/kg DM (with a rumen outflow rate of 4.4%/h and a degradation rate of 0.180/h (evaluated in adult cows); [6] 16.1 kJ gross energy/g; [7] 389.5 g non starch polysaccharides/kg; 51.9 g ADF/kg; 0.35 g ADL/kg; 14.5 mg sucrose/g.

Table S3. Chemical composition (g/kg DM) of varieties of faba bean.

Cultivar	CP	EE	CF	NDF	ADF	ADL	Starch
Portuguese[1]							
Favel [44, 110]	255	-	-	204	103	21	409
Others[2]							
cv. Beja[3] [78]	237	14	100	202	131	24	400
Unknown [82]	256	-	-	106	69	-	358
Unknown[4] [105]	270	-	14	-	-	-	-
Unknown[5] [83]	243	19	-	152	94	6	224
Mean	252	17	57	153	98	15	327
SD	14.7	3.5	60.8	48.0	31.2	12.7	91.9

ADF, acid detergent fibre; ADL, acid detergent fibre; CF, crude fibre; CP, crude protein; DM, dry matter; EE, ether extract; NDF, neutral detergent fibre.

[1] Included in the CNV [31]; [2] Portuguese varieties not included in the CNV; [3] 917 g organic matter digestibility/kg DM; 16.3 MJ digestible energy/kg DM (evaluated in Merino rams); [4] 16.7 kJ gross energy/g; 14 g crude fibre/kg; [5] 435.5 g non starch polysaccharides/kg; 17.2 mg sucrose/g.

Table S4. Chemical composition (g/kg DM) of varieties of lupins.

Cultivar	Ash	CP	EE	CF	NDF	ADF	Starch	NFE
Portuguese[1]								
White lupin								
Estoril (sweet) [110]	38	363	-	-	231	156	n.d.	-
Yellow lupin								
Cardiga (bitter)[3] [47, 78]	47	381	60	202	286	246	7	231
Others[2]								
Sweet white lupin								
804-4 [117]	29	374	120	117	-	-	-	360
930-3 [117]	31	363	116	121	-	-	-	369
551-3 [117]	31	373	116	118	-	-	-	362
802-15 [117]	31	362	117	106	-	-	-	384
816-20 [117]	30	394	116	108	-	-	-	353
968-12 [117]	32	376	114	103	-	-	-	376
551-5 [117]	33	350	124	121	-	-	-	372
893-7 [117]	31	372	104	120	-	-	-	373
357-2 [117]	33	370	115	113	-	-	-	370
Unknown [82]	-	339	-	-	212	153	-	-
Mean	31	367	116	114	-	-	-	369
SD	1.3	15.1	5.4	6.9	-	-	-	9.3
Blue lupin								
Illyarie[4] [78]	26	291	72	161	269	229	8	-
8176[5] [79]	40	342	77	-	271	-	11	-
Unknown[6] [105]	23	327	-	63	-	-	-	-
Unknown[7] [83]	-	338	61	-	197	138	0	-
Unknown[8] [108]	28	340	64	-	-	-	-	-
Mean	29	328	69	112	246	184	6	-
SD	7.5	21.3	7.3	69.3	42.2	64.3	5.7	-
Yellow lupin								
Refusa (sweet) [47]	52	437	66	-	-	-	-	200
RM 102-B (sweet) [47]	48	417	58	-	-	-	-	199
RM 202-B [47]	59	456	60	-	-	-	-	191
RM 202-P (bitter) [47]	52	433	58	-	-	-	-	201
Unknown[9] [24]	46	425	48	-	-	-	15	321
3	51	434	58	-	-	-	-	222
Mean								
SD	5.0	14.7	6.5	-	-	-	-	55.3

CF, crude fibre; CP, crude protein; DM, dry matter; EE, ether extract; NDF, neutral detergent fibre; NFE, N free extract; n.d., not detected.

[1] Included in the CNV [31]; [2] Portuguese varieties not included in the CNV; [3] 28 g acid detergent lignin (ADL)/kg DM, 836 g organic matter digestibility (OMD)/kg DM, 16.9 MJ digestible energy (DE)/kg DM (evaluated in Merino rams); [4] 28 g ADL/kg DM, 860 g OMD/kg DM, 16.5 MJ DE/kg DM (evaluated in Merino rams); [5] 798 g degradable DM/kg (with a rumen outflow rate of 4.4%/h and a degradation rate of 0.135/h) and 856 g degradable nitrogen/kg DM (with a rumen outflow rate of 4.4%/h and a degradation rate of 0.166/h); [6] 18.4 kJ gross energy/g; [7] 477.5 g non starch polysaccharides/kg; 5.45 g ADL/kg; 31.7 mg sucrose/g; [8] 17.6 kJ energy/g; [9] 125 g pentosans/kg DM; 161 g crude cellulose/kg DM; 3 g lignin/kg DM.

CHAPTER 3: PROXIMATE AND PHYTOCHEMICAL COMPOSITION OF EUROPEAN VARIETIES OF GRAIN LEGUMES

PROXIMATE AND PHYTOCHEMICAL COMPOSITION OF EUROPEAN VARIETIES OF GRAIN LEGUMES[1]

1. Introduction

Grain legumes (GL), also called pulses, are crops of the botanical family *Fabaceae*. Examples of GL used for food and feed include, for example, chickpeas (*Cicer arietinum* L.), field peas (*Pisum sativum* L.), faba beans (*Vicia faba* L.), lupins (*Lupinus* spp. L.), vetches (e.g. common vetch, *Vicia sativa* L. or chickling vetch, *Lathyrus cicera* L.), common beans (*Phaseolus vulgaris* L.) or lentils (*Lens lens* L.). As good sources of crude protein [CP; 1], GL constitute appealing economical and sustainable alternatives to the protein sources commonly used in animal feedstuffs such as soybean meal (SBM) and fishmeal [2, 3]. Additionally, legume seeds are good sources of energy and fibre [2, 4] also presenting non-nutrients, product of plants' secondary metabolism, that may exert positive, negative or both effects when ingested [5]. Non-nutrients, particularly those with antinutritional effect, are of greater concern to monogastrics than for ruminants because these latter can destroy or modify these metabolites through rumen fermentation [4, 6]. Nonetheless, GL varieties with negligible or low amounts of antinutritional factors are preferred for both classes of animals [6, 7].

Despite the low European production of GL due to the reasons previously address in this dissertation (*Section 1.1.2.2*), European countries present suitable edaphoclimatic conditions to cultivate these crops and measures towards increasing their local production have already been purposed by the European Commission as a way of decreasing the external dependence on soybeans and SBM [8]. The year of 2016 was even declared as the International Year of Pulses by the 68th United Nations General Assembly [9]. It is therefore, crucial to fully characterize these locally-produced ingredients for an adequate inclusion in the diets of different farmed animals.

In this context, the aim of the work presented in this chapter was to characterize in depth marketable European GL varieties in terms of proximate composition and profiles in fatty acids (FA), carotenoids and organic acids. The phytochemical profile regarding phenolic compounds and alkaloids of some of the varieties herein characterized was recently determined [10-12].

[1] This work had the collaboration of Margarida R.G. Maia (ICBAS-UP), Ana R.J. Cabrita (ICBAS-UP), Patrícia Valentão (FFUP), Paula B. Andrade (FFUP) and António J.M. Fonseca (ICBAS-UP). The manuscript is still under preparation and has not been validated by coauthors.

2. Material and methods

2.1. Sampling

A total of 51 GL seeds (registered in the European Plant Variety Database [13] except yellow lupin *Lupinus luteus*, cv. Nacional), offered by the seed companies Agri-Obtentions (Guyancourt, France), Agroservice SpA (S. Severino Marche, Italy), Fertiprado (Vaiamonte, Portugal), Florimond Desprez (Cappelle-en-Pévèle, France), Institute of Plant Genetics of the Polish Academy of Sciences (Poznan, Poland), Instituto Nacional de Investigação Agrária e Veterinária, I.P. (Oeiras, Portugal), Jouffray-Drillaud (Vienne, France), RAGT Seeds Ltd (Saffron Walden, UK) and Semillas El Solc S.L. (Lleida, Spain), included mature raw whole seeds of Kabuli (CHK; large, white to cream seeds; n=5) and Desi (CHD; small and dark seeds; n=1) chickpeas, field peas (FP; n=21), faba beans (FB; n=10), white lupins (WL; *Lupinus albus*; n=5), narrow-leafed lupins (NLL; *L. angustifolius*; n=2), yellow lupins (YL; n=5), common vetch (CoV; n=1) and chickling vetch (CV; n=1; Table 1). After reception, seeds were dried in a forced-air oven (65 ºC, 24 h) and grounded to 1 mm for further analysis (0.5 mm for starch).

2.2. Proximate composition

All 51 GL seed varieties were analyzed for proximate composition, total lipids and FA profile whereas only 30 were studied for organic acids and carotenoids evaluation. These 30 samples are clearly identified also in Table 1 and included all the varieties belonging to the Portuguese catalog of varieties [n=12; 14] as well as others that, besides not Portuguese, were grown in the country (n=3); additionally, depending on seed availability, other varieties were added to the analysis (n=15).

According to AOAC [15], dry matter (DM) of samples was determined after drying at 103 ± 2 ºC for 2 h (method 930.15), ash was obtained after incineration at 550 ± 20 ºC for 3 h and CP was calculated as 6.25 x Kjeldahl N (method 954.01). Soluble CP, total lipids and starch were determined according to Hart and Bentley [16], Folch *et al.* [17] and Salomonsson *et al.* [18], respectively. Neutral detergent fibre (NDF; assayed with heat stable amylase and expressed exclusive of residual ash), acid detergent fibre (ADF; expressed inclusive of residual ash) and acid detergent lignin (ADL; determined by solubilization of cellulose with sulphuric acid and expressed exclusive of residual ash) contents were determined by the procedures of Van Soest *et al.* [19] and Robertson and Van Soest [20]. Gross energy (GE) content was determined in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany). All analysis were run in duplicate.

Table 1. List of varieties and respective supplier countries of the studied grain legume samples.

Material	Origin	Material	Origin
Chickpea		*Field pea*	
Elmo (Desi type)*	Portugal	Cartouche	France
Eldorado (Kabuli type)*	Portugal	Cherokee	France
Elixir (Kabuli type)*	Portugal	Cigal	France
Elvar (Kabuli type)*	Portugal	Comanche	France
Reale (Kabuli type)*	Italy	Dove	France
Sultano (Kabuli type)*	Italy	Eiffel	Italy
		Enduro	France
Faba bean var. minor		Esmeralda*	Spain
Chiaro di Torrelama*	Italy	Genial	France
Diva*	France	Grisel*	Portugal
Fabelle*	France	Indiana	France
Favel*	Portugal	Isard	France
Gladice	France	James	France
Irena	France	Marqueta*	Spain
Nordica	France	Montrebei*	Spain
Organdi*	France	Montsant*	Spain
Rumbo	Italy	Pixel-I*	Portugal
Scuro di Torrelama*	Italy	Pixel-L	Portugal
		Spacial	France
White lupin		Standal	France
Amiga*	France	Verbal	France
Estoril*	Portugal		
Lumen*	France	*Narrow-leafed lupin*	
Multitalia-IT	Italy	Azuro*	Portugal
Multitalia-PT*	Portugal	Sonet*	Poland
Yellow lupin		*Chickling vetch*	
Dukat*	Poland	Grão-da-gramicha*	Portugal
Mister-PL	Poland		
Mister-PT*	Portugal	*Common vetch*	
Nacional*	Portugal	Barril*	Portugal
Taper*	Poland		

*Varieties analyzed for carotenoids and organic acids profiles in addition to proximate composition and fatty acids profile.

2.3. Fatty acids composition

Lipids from dried seeds were extracted by a modified procedure of Folch *et al.* [17], using a dichloromethane:methanol (2:1, v/v) solution and determined gravimetrically. Fatty acid methyl esters (FAME) were prepared by direct transesterification following Alves *et al.* [21] and heptadecanoic acid (C17:0; Sigma-Aldrich, St. Louis, MO; 0.5 mg/mL) was used as the internal standard.

The FAME were analyzed by gas chromatography (GC) using a GC-2010 Plus (Shimadzu Europe GmbH, Germany) chromatograph equipped with a flame ionization detector and a fused-silica capillary column (Omegawax 250, 30 m × 0.25 mm × 0.25 µm; Supelco, Bellefonte, PA). Helium was the carrier gas, and the split ratio was 1:100. The injector and detector temperatures were 250 and 260°C, respectively. The initial oven temperature of 150°C was held for 7 min, increased at 3°C/min to 170°C and held for 25 min, and then increased at 3ºC/min to 220ºC and held for 30 min. Peak identification was based on comparison of retention times with FAME standards (Supelco 37 component FAME mix, Sigma-Aldrich, St, Louis, MO; GLC-110 mixture and Bacterial acid methyl esters CP mixture, Matreya LLC, Pleasant Gap, PA). Analysis were run in duplicate

2.4. Secondary compounds

The procedure used to determine GL seeds carotenoids and organic acids were as previously described by Fernandes et al. [22] and Magalhães et al. [23], respectively.

2.5. Statistical analysis

A discriminant analysis was performed (SPSS®, v.24; IBM, USA) on the 51 GL samples using seeds' CP content as the categorical dependent variable (Table 2). Grain legumes were grouped according to species being further divided in two different groups (G1 and G2) according to their CP content in order to create distinct ranges of CP values (Table 2). Both vetches (common and chickling ones) were included together with faba beans given the overall similarities they presented in terms of chemical composition.

Table 2. Discriminant analysis applied on grain legume groups using seeds CP content as the categorical dependent variable.

Grain legume group	Group	CP range, g CP/100 g DM	n
Chickpeas	G1	21.0 - 22.1	3
	G2	23.7 - 27.0	3
Field peas	G1	21.3 - 23.0	11
	G2	23.1 - 26.9	10
Faba beans, common vetch and chickling vetch	G1	22.3 - 26.8	6
	G2	28.1 - 32.8	6
White, narrow-leafed and yellow lupins	G1	26.8 - 36.5	5
	G2	38.0 - 42.6	7

Mean values of chemical parameters analyzed in the seeds, i.e. independent variables (ash, NDF, ADF, ADL, GE, soluble CP, lipids, total and individual FA and SFA, MUFA and

PUFA contents), were compared between quartiles (within a group) by one-way analysis of variance. Tukey's HSD *post hoc* test was used to compare means. In all cases, significant differences were considered when $P < 0.05$.

3. Results

3.1. Proximate composition of grain legumes

Proximate composition and GE content of all studied grain legume varieties is presented in Table 3. Average CP content was highest for lupins, in particular for YL varieties (40 ± 1.9 g/100 g DM) which were followed by WL and NLL (37 ± 1.1 and 30 ± 3.1 g CP/100 g DM, respectively) and lowest for CHD and CV (ca. 22.0 g/100 g DM). The varieties with the lowest and highest levels of CP within each species were as follows: respectively, Eldorado and Sultano for CHK, Enduro and Montrebei for FP, Nordica and Fabelle for FB, Lumen and Multitalia-IT for WL and Mister-PT and Taper for YL. The solubility of seeds' protein was high having ranged, in average, between 50.3 g/100 g CP in CV and 66.1 g/100 CP in WL samples. Neutral detergent fibre and ADF contents were also highest for lupins: respectively, 30.3 and 20.0 g/100 g DM for NLL, 27.2 and 18.8 g/100 g DM for YL and 24.8 and 16.9 g/100 g DM for WL; the lowest values were found in CHK samples (13.5 and 3.2 g/100 g DM, respectively). ADL contents varied between 0.6 g/100 g DM in FP and 2.4 g/100 g DM in YL. Seeds' starch levels ranged from 27.3 g/100 g DM in CHD to 40.4 g/100 g DM in CoV and were null in all lupin samples. In average, highest GE values were found in lupins and CHK (17.5-18.7 MJ/kg DM) whereas in all the other legume samples GE levels ranged between 16.0 and 16.4 MJ/kg DM.

3.2. Total lipid content and fatty acids profile of grain legumes

Grain legume varieties' total content in lipids as well as their profiles in FA can be observed in Table 4 (the complete individual FA profile of each sample is presented as supplementary material in Table S1). A chromatogram of a GL variety (CHK var. Elvar) is shown in Figure 1.

Lipids were found at higher contents in WL (9.1 g/100 g DM) than in all the other legume samples (2.4-6.4 g/100 g DM in vetches and CHK, respectively).

Table 3. Proximate composition and gross energy content of the grain legume samples.[1]

Samples	Ash	CP[2]	Soluble CP	NDF	ADF	ADL	Starch	GE
	g/100 g DM		g/100 g CP	g/100 g DM				MJ/kg DM
Chickpea								
Elmo	3.4	22.0	50.3	22.9	9.4	1.5	27.3	16.4
Eldorado	3.0	21.0	63.7	13.3	3.0	2.2	35.5	17.4
Elixir	2.8	23.7	63.5	12.5	3.0	1.7	37.5	17.5
Elvar	3.0	22.1	60.4	15.8	3.2	1.9	36.6	17.2
Reale	3.2	26.3	67.7	13.3	3.4	0.3	30.5	17.9
Sultano	3.3	27.1	63.8	12.3	3.3	1.0	31.7	17.5
Mean[3]	**3.1**	**24.0**	**63.8**	**13.5**	**3.2**	**1.4**	**34.4**	**17.5**
SD[4]	**0.18**	**2.34**	**2.45**	**1.30**	**0.22**	**0.70**	**2.78**	**0.21**
Field pea								
Cartouche	2.9	23.4	66.3	18.4	7.4	0.7	40.0	16.5
Cherokee	2.7	22.4	59.3	19.1	7.2	0.2	39.7	16.4
Cigal	3.0	24.0	67.4	16.5	7.0	0.8	37.5	16.4
Comanche	3.0	23.0	65.8	18.4	7.2	0.5	40.6	16.3
Dove	3.4	21.4	62.6	20.9	7.7	0.5	39.7	16.1
Eiffel	3.0	22.3	64.6	18.0	7.0	0.3	41.5	16.2
Enduro	3.1	21.3	60.2	22.1	7.3	0.2	39.0	16.0
Esmeralda	2.7	25.1	56.9	19.6	7.4	0.9	34.9	16.4
Genial	3.1	23.0	65.1	20.4	6.8	0.7	44.6	16.2
Grisel	3.3	23.1	66.1	18.7	7.6	1.8	40.5	15.9
Indiana	2.6	21.4	71.9	16.6	6.5	0.7	42.0	16.3
Isard	2.8	22.2	64.1	20.8	7.7	0.4	38.5	16.0
James	2.9	23.5	53.8	21.3	6.5	0.7	38.3	16.2
Marqueta	3.0	24.4	56.9	19.7	7.1	0.3	37.7	16.2
Montrebei	3.3	26.9	57.4	20.4	7.0	0.5	37.2	16.5
Montsant	3.1	22.7	52.6	20.3	7.3	1.4	40.0	16.3
Pixel-I	3.3	23.6	62.5	19.3	6.6	0.6	37.0	15.8
Pixel-L	3.1	24.0	66.1	17.5	7.5	0.6	40.7	16.1
Spacial	3.0	21.7	63.7	17.6	6.1	0.3	43.6	16.4
Standal	2.7	22.6	66.8	19.6	6.6	0.2	39.4	16.0
Verbal	3.2	23.9	62.3	21.4	7.9	0.9	37.3	16.0
Mean	**3.0**	**23.1**	**62.5**	**19.4**	**7.1**	**0.6**	**39.5**	**16.2**
SD	**0.22**	**1.33**	**4.81**	**1.57**	**0.47**	**0.40**	**2.25**	**0.20**
Faba bean								
Chiaro di Torrelama	3.3	26.8	59.5	20.3	10.1	0.4	37.4	16.5
Diva	4.1	29.7	66.3	18.4	9.6	0.7	32.1	16.3
Fabelle	4.2	32.8	62.2	17.9	9.5	1.5	31.4	16.1
Favel	4.0	25.0	63.1	20.5	10.3	2.1	38.4	15.9
Gladice	3.4	29.9	67.7	19.1	9.0	0.9	32.6	16.5

Irena	4.4	28.1	57.2	20.7	10.8	1.5	32.1	16.3
Nordica	3.3	25.6	66.3	20.2	9.4	1.3	35.8	16.2
Organdi	3.4	29.1	66.2	21.0	12.0	1.5	33.8	16.5
Rumbo	3.7	25.7	58.8	22.6	10.3	0.5	36.8	16.2
Scuro di Torrelama	3.2	31.6	65.6	17.5	9.9	1.4	34.6	16.7
Mean	**3.7**	**28.4**	**63.0**	**19.8**	**10.1**	**1.2**	**34.5**	**16.3**
SD	**0.41**	**2.52**	**4.32**	**1.51**	**0.84**	**0.60**	**2.39**	**0.23**
White lupin								
Amiga	3.6	36.5	62.4	25.1	16.7	1.2	n.d.[5]	19.2
Estoril	3.8	36.0	67.5	23.1	15.6	0.8	n.d.	18.3
Lumen	3.8	35.7	70.7	25.0	18.0	1.1	n.d.	18.5
Multitalia-IT	3.3	38.3	62.9	24.0	16.4	1.1	n.d.	19.1
Multitalia-PT	4.1	38.0	67.1	26.9	17.9	1.6	n.d.	18.3
Mean	**3.7**	**36.9**	**66.1**	**24.8**	**16.9**	**1.2**	**-**	**18.7**
SD	**0.29**	**1.05**	**3.13**	**1.29**	**0.96**	**0.31**	**-**	**0.41**
Narrow-leafed lupin								
Azuro	3.1	32.9	63.8	30.1	21.0	1.7	n.d.	17.6
Sonet	4.1	26.8	54.8	30.6	19.1	2.9	n.d.	17.5
Mean	**3.6**	**29.8**	**59.3**	**30.3**	**20.0**	**2.3**	**-**	**17.5**
SD	**0.50**	**3.05**	**4.50**	**0.24**	**1.01**	**0.61**	**-**	**0.06**
Yellow lupin								
Dukat	5.6	41.9	67.8	26.3	18.2	3.2	n.d.	17.9
Mister-PL	5.5	40.8	63.1	26.9	18.9	3.5	n.d.	17.7
Mister-PT	5.0	38.0	66.1	28.2	19.4	1.3	n.d.	17.9
Nacional	5.8	38.3	63.9	29.1	20.0	1.1	n.d.	17.7
Taper	5.6	42.6	67.0	25.7	17.7	2.8	n.d.	17.8
Mean	**5.5**	**40.3**	**65.6**	**27.2**	**18.8**	**2.4**	**-**	**17.8**
SD	**0.29**	**1.86**	**1.83**	**1.26**	**0.86**	**1.03**	**-**	**0.11**
Chickling vetch								
Grão-da-gramicha	3.7	22.3	50.3	22.6	9.2	1.4	34.7	16.1
Common vetch								
Barril	3.7	25.3	59.8	18.7	6.0	1.3	40.4	16.0

[1]Results expressed as mean values (n=2). [2]NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; CP, crude protein; GE, gross energy. [3]Mean values of Kabuli type varieties (all except var. Elmo). [4]SD; standard deviation. [5]nd, not detected.

Within FA, five were considered major FA given their proportions in the seeds when compared to the other FA, namely, C16:0 (palmitic acid), C18:0 (stearic acid), C18:1*c*9 (oleic acid), C18:2*n*6 (linoleic acid) and C18:3*n*3 (α-linolenic acid). These FA together accounted at least for 84% of total FA in the GL seeds. C16:0 average content was lowest for YL (6.7 g/100 g FA) followed by WL (8.8 g/100 FA) and highest for CV (16.7 g/100 g FA). C18:0 was lowest for CHD and WL (< 1.8 g/100 FA) and highest for NLL and CoV (> 6.4 g/100 g FA).

C18:1c9 was highest for WL (50.6 g/100 g FA) and lowest for CoV (12.0 g/100 g FA) whereas C18:2*n*6 was found at highest and lowest levels in CHD and WL, respectively (59.2 and 17.6 g/100 g FA, respectively). Finally, C18:3*n*3 varied, in average, from 2.2 g/100 g FA in CHK to 8.3 g/100 g FA in FP. Overall, PUFA (47.0-61.9 g/100 g FA) predominated over SFA and MUFA in all legumes seeds except in WL which, instead, presented increased levels of MUFA (57.6 g/100 g FA). *n*6/*n*3 FA ratio was highest for chickpeas, followed by FB, and lowest for WL (in average 23.1, 17.1 and 2.3, respectively).

3.3. Carotenoids and organic acids profiles of grain legumes

Seeds carotenoids and organic acids profiles are presented in Table 5 and chromatograms of one chickpea variety type Kabuli (var. Elvar) can be seen in Figures 2 and 3, respectively.

Among all varieties, only two carotenoids were identified, namely lutein and zeaxanthin (both xanthophylls). Lutein was present in all samples (0.1-0.8 mg/100 g DM) whereas zeaxanthin was only found in chickpeas and lupins (1.6-15.4 mg/100 g DM). Among all chickpeas, CHD stood out from the CHK ones in terms of total carotenoids content (16.2 vs. 6.4 mg/100 g DM, respectively). For the other grain legumes species, total carotenoids content was lower (0.2-3.3 mg/100 g DM).

Several organic acids were identified in grain legume seeds, namely, oxalic, aconitic, citric, pyruvic, malic and fumaric acids. Citric and aconitic acids were common to all varieties, the former being the major compound in all samples, varying, in average, from 23.4 mg/100 g DM in CoV to 385.1 mg/100 g DM in WL. Consequently, WL presented the highest total amount of organic acids (404 mg/100 g DM) and CoV the lowest (55 mg/100 g DM). Oxalic acid was absent in all FP and FB varieties and varied between 2.0 and 7.7 mg/100 g DM in the other species.

Table 4. Total lipids content and fatty acids composition (dry matter basis) of the grain legume samples.[1]

Samples	Lipids	FA[2]	C16:0	C18:0	C18:1*c*9	C18:2*n*6	C18:3*n*3	SFA	MUFA	PUFA	n6/n3
	g/100 g DM					g/100 g FA					
Chickpea											
Elmo	4.2	4.0	12.6	1.4	19.4	59.2	2.9	16.1	21.5	61.9	20.2
Eldorado	6.5	6.0	11.0	1.6	24.0	57.0	2.4	14.5	25.8	59.2	23.3
Elixir	6.3	5.6	10.7	1.5	28.6	52.7	2.4	14.0	30.6	54.8	21.9
Elvar	7.3	5.7	10.8	1.6	25.5	55.5	2.5	14.2	27.4	57.7	21.9
Reale	6.1	5.1	9.7	7.1	34.3	42.8	1.8	20.1	35.2	44.3	24.0
Sultano	5.8	5.1	10.3	1.4	35.5	46.8	1.9	13.6	37.6	48.5	24.4
Mean[3]	**6.4**	**5.5**	**10.5**	**2.6**	**29.6**	**51.0**	**2.2**	**15.3**	**31.3**	**52.9**	**23.1**
SD[4]	**0.44**	**0.36**	**0.48**	**2.23**	**4.60**	**5.35**	**0.31**	**2.45**	**4.48**	**5.64**	**1.17**
Field pea											
Cartouche	7.0	1.8	13.2	4.0	25.7	45.5	8.6	19.0	26.5	53.9	5.2
Cherokee	3.0	1.5	14.4	2.9	22.4	47.0	10.2	19.0	23.3	57.1	4.5
Cigal	4.1	1.6	14.2	4.2	25.7	42.6	10.0	20.5	26.6	52.4	4.2
Comanche	3.0	1.5	14.5	3.5	17.0	53.0	9.1	19.9	17.8	61.9	5.8
Dove	4.2	1.5	13.5	4.1	24.5	47.3	7.0	19.4	25.6	54.2	6.6
Eiffel	3.4	1.3	12.9	3.6	26.6	43.8	10.2	18.2	27.5	53.8	4.3
Enduro	2.5	1.6	15.1	3.1	18.5	52.8	7.9	20.0	19.0	60.5	6.6
Esmeralda	3.9	1.3	14.6	3.2	20.0	49.6	9.1	19.9	21.0	58.4	5.4
Genial	3.4	1.2	13.3	4.9	19.3	51.7	7.6	20.3	20.2	59.1	6.7
Grisel	2.8	1.4	13.2	3.0	23.7	49.3	7.3	18.1	24.6	56.5	6.6
Indiana	3.1	1.4	15.3	3.2	24.3	44.1	9.9	20.3	25.3	53.8	4.4
Isard	2.7	1.2	13.3	3.2	21.4	50.6	8.6	18.1	22.0	59.2	5.7
James	3.3	1.1	14.9	3.1	15.5	53.0	9.8	20.0	16.6	62.6	5.3
Marqueta	3.7	1.5	14.4	3.8	21.4	49.2	7.7	20.4	22.2	56.7	6.3
Montrebei	2.7	1.2	14.9	4.0	20.9	49.1	7.9	21.0	21.8	56.7	6.2
Montsant	3.1	1.4	14.4	3.8	20.5	50.1	7.6	20.3	21.4	57.5	6.4
Pixel-I	3.5	1.6	12.4	3.1	26.6	46.3	8.5	17.2	27.6	54.6	5.3
Pixel-L	3.7	1.7	14.0	3.8	37.0	36.2	5.8	19.7	38.0	41.8	6.1
Spacial	3.5	1.2	12.6	4.0	19.3	53.0	7.9	18.3	20.4	60.7	6.6
Standal	3.4	1.3	13.1	4.3	28.9	42.9	7.2	19.1	30.1	50.0	5.8
Verbal	2.5	1.3	13.3	3.5	27.7	45.0	7.4	18.5	28.8	52.3	6.0
Mean	**3.5**	**1.4**	**13.9**	**3.6**	**23.2**	**47.7**	**8.3**	**19.4**	**24.1**	**55.9**	**5.7**
SD	**0.93**	**0.19**	**0.85**	**0.51**	**4.70**	**4.23**	**1.17**	**0.99**	**4.76**	**4.56**	**0.80**
Faba bean											
Chiaro di Torrelama	2.7	1.3	16.2	2.3	21.8	52.1	3.2	21.7	22.6	55.1	15.9
Diva	3.6	1.4	13.7	2.4	24.9	52.4	2.6	18.8	25.8	54.8	19.1
Fabelle	2.5	1.3	13.6	1.9	21.0	56.3	3.0	18.2	21.9	59.2	18.0
Favel	2.7	1.5	15.6	2.5	23.5	50.4	3.3	21.6	24.4	53.4	15.1

Gladice	3.4	1.4	15.0	2.7	26.9	47.7	2.7	20.9	27.9	50.3	16.4
Irena	3.9	1.5	14.3	2.1	25.6	50.6	3.1	19.4	26.5	53.6	15.8
Nordica	2.5	1.5	14.8	2.0	24.0	52.6	2.5	19.5	24.9	55.0	19.7
Organdi	3.3	1.3	15.4	2.8	24.4	49.2	3.0	21.5	25.5	52.1	15.4
Rumbo	3.3	1.4	15.1	2.4	25.9	49.6	2.9	20.5	26.8	52.3	16.8
Scuro di Torrelama	3.0	1.2	15.7	2.2	22.1	52.6	2.7	21.0	23.1	55.1	18.6
Mean	**3.1**	**1.4**	**14.9**	**2.3**	**24.0**	**51.4**	**2.9**	**20.3**	**24.9**	**54.1**	**17.1**
SD	**0.52**	**0.10**	**0.82**	**0.28**	**1.82**	**2.28**	**0.24**	**1.18**	**1.83**	**2.28**	**1.57**
White lupin											
Amiga	9.7	9.0	7.7	1.5	51.0	18.6	8.8	14.0	58.1	27.5	2.1
Estoril	10.7	7.5	8.4	1.7	47.8	19.1	7.7	15.5	56.7	27.1	2.5
Lumen	8.3	7.6	8.9	1.9	55.7	13.6	7.2	16.3	62.4	20.8	1.9
Multitalia-IT	8.8	7.3	9.1	1.8	47.9	18.9	7.3	16.6	56.0	26.5	2.6
Multitalia-PT	8.1	5.5	9.9	1.6	47.6	17.8	8.2	17.9	54.9	26.2	2.2
Mean	**9.1**	**7.4**	**8.8**	**1.7**	**50.6**	**17.6**	**7.8**	**16.1**	**57.6**	**25.6**	**2.3**
SD	**0.99**	**1.14**	**0.74**	**0.14**	**3.12**	**2.07**	**0.57**	**1.28**	**2.60**	**2.44**	**0.27**
Narrow-leafed lupin											
Azuro	6.1	4.3	11.6	6.2	27.9	44.5	5.0	21.4	28.9	49.2	8.8
Sonet	6.0	5.5	10.2	6.8	33.7	40.2	4.8	20.4	34.5	44.8	8.4
Mean	**6.1**	**4.9**	**10.9**	**6.5**	**30.8**	**42.4**	**4.9**	**20.9**	**31.7**	**47.0**	**8.6**
SD	**0.20**	**0.58**	**0.70**	**0.30**	**2.91**	**2.13**	**0.09**	**0.50**	**2.82**	**2.23**	**0.26**
Yellow lupin											
Dukat	6.2	5.1	6.6	3.0	21.8	48.2	7.4	18.7	24.8	55.9	6.5
Mister-PL	6.0	4.9	6.2	2.7	23.5	48.9	6.6	16.8	26.7	55.7	7.5
Mister-PT	4.7	3.6	7.2	2.6	30.4	41.8	4.8	18.0	34.1	46.8	8.6
Nacional	5.4	5.0	7.1	2.3	20.4	50.1	6.6	16.6	25.5	56.9	7.6
Taper	5.5	4.9	6.5	2.7	21.1	49.9	8.1	17.1	24.0	58.2	6.2
Mean	**5.6**	**4.7**	**6.7**	**2.7**	**23.4**	**47.8**	**6.7**	**17.5**	**27.0**	**54.7**	**7.3**
SD	**0.55**	**0.61**	**0.36**	**0.22**	**3.63**	**3.06**	**1.09**	**0.78**	**3.67**	**4.04**	**0.85**
Chickling vetch											
Grão-da-gramicha	2.4	1.0	16.7	6.7	12.0	50.8	6.7	27.1	13.0	59.3	7.6
Common vetch											
Barril	2.4	1.2	15.5	3.5	12.0	54.3	6.1	22.1	16.5	60.6	8.3

[1]Results expressed as mean values (n=2). [2]DM, dry matter; FA, fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids. [3]Mean values of Kabuli type varieties (all except var. Elmo). [4]SD; standard deviation.

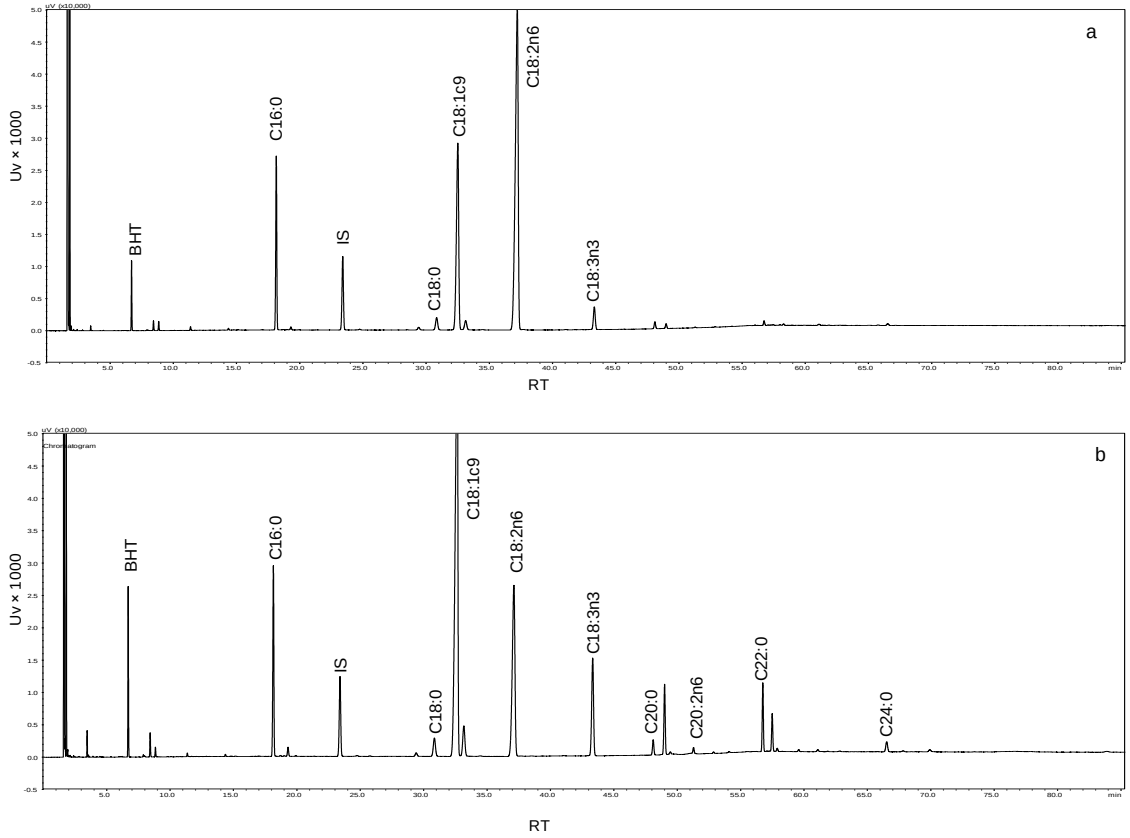

Figure 1. **GC-FID fatty acids profile of a) chickpea type Kabuli var. Elvar and b) white lupin var. Estoril. BHT, butylated hydroxytoluene; IS, internal standard (C17:0); RT, retention time.**

Table 5. Carotenoids and organic acids composition (mg/100 g, dry matter basis) of the grain legume samples.[1]

Samples	Carotenoids			Organic acids						
	Lutein	Zeaxanthin	Total	Oxalic	Aconitic	Citric	Pyruvic	Malic	Fumaric	Total
Chickpea										
Elmo	0.81	15.42	16.23	6.1	0.3	110.1	n.d.[4]	22.3	0.1	138.9
Eldorado	0.32	8.44	8.76	2.4	0.5	154.9	n.d.	13.2	n.d.	171.0
Elixir	0.25	8.02	8.28	7.2	1.1	255.7	n.d.	30.6	n.d.	294.5
Elvar	0.31	8.10	8.41	6.5	0.5	324.6	n.d.	30.9	0.1	362.6
Reale	0.20	4.85	5.05	2.9	0.4	181.4	n.d.	27.5	n.d.	212.2
Sultano	0.10	2.77	2.87	2.0	0.3	186.0	n.d.	23.2	n.d.	211.6
Mean[2]	**0.24**	**6.44**	**6.67**	**4.2**	**0.5**	**220.5**	**-**	**25.1**	**0.1**	**250.4**
SD[3]	**0.083**	**2.252**	**2.333**	**2.18**	**0.24**	**62.30**	**-**	**6.58**	**0.04**	**69.42**
Field pea										
Esmeralda	0.49	n.d.	0.49	n.d.	0.4	88.7	1.9	28.5	0.1	119.6
Grisel	0.36	n.d.	0.36	n.d.	0.2	104.0	n.d.	33.5	n.d.	137.7
Marqueta	0.36	n.d.	0.36	n.d.	0.6	243.9	2.7	28.2	0.1	275.4
Montrebei	0.90	n.d.	0.90	n.d.	1.4	104.3	3.2	30.0	0.1	139.0
Montsant	0.39	n.d.	0.39	n.d.	0.8	86.6	1.3	13.2	0.1	101.9
Pixel-I	0.43	n.d.	0.43	n.d.	0.3	97.6	n.d.	25.7	nd	123.7
Mean	**0.49**	**-**	**0.49**	**-**	**0.6**	**120.8**	**1.5**	**26.5**	**0.1**	**149.5**
SD	**0.190**	**-**	**0.190**	**-**	**0.38**	**55.81**	**1.22**	**6.50**	**0.04**	**57.95**
Faba bean										
Chiaro di Torrelama	0.33	n.d.	0.33	n.d.	1.3	114.6	23.8	39.4	0.1	179.1
Diva	0.24	n.d.	0.24	n.d.	1.3	60.8	13.9	28.2	n.d.	104.1
Fabelle	0.39	n.d.	0.39	n.d.	0.5	151.7	4.0	49.1	0.1	205.3
Favel	0.36	n.d.	0.36	n.d.	1.3	97.2	22.6	56.7	n.d.	177.7
Organdi	0.29	n.d.	0.29	n.d.	1.7	138.6	32.7	36.9	n.d.	209.8
Scuro di Torrelama	0.21	n.d.	0.21	n.d.	1.5	116.9	13.4	33.7	n.d.	165.4
Mean	**0.30**	**-**	**0.30**	**-**	**1.2**	**113.3**	**18.4**	**40.6**	**0.0**	**173.6**
SD	**0.065**	**-**	**0.065**	**-**	**0.34**	**29.36**	**9.19**	**9.58**	**0.05**	**34.89**
White lupin										
Amiga	0.05	1.43	1.48	7.7	0.3	393.5	n.d.	8.4	0.1	410.0
Estoril	0.12	1.78	1.89	4.7	0.3	362.5	n.d.	12.9	n.d.	380.4
Lumen	0.16	1.67	1.84	6.9	0.3	347.2	n.d.	9.8	0.1	364.2
Multitalia-PT	0.18	1.40	1.58	5.9	0.3	437.4	n.d.	18.0	0.1	461.6
Mean	**0.13**	**1.57**	**1.70**	**6.3**	**0.3**	**385.1**	**-**	**12.3**	**0.1**	**404.0**
SD	**0.051**	**0.168**	**0.178**	**1.13**	**0.01**	**34.50**	**-**	**3.69**	**0.02**	**37.09**
Narrow-leafed lupin										
Azuro	0.15	2.14	2.29	3.4	0.4	182.2	2.0	18.8	0.1	206.8
Sonet	0.24	4.01	4.25	0.7	0.2	121.9	1.6	9.4	n.d.	133.8
Mean	**0.20**	**3.07**	**3.27**	**2.0**	**0.3**	**152.0**	**1.8**	**14.1**	**-**	**170.3**

SD	**0.044**	**0.939**	**0.982**	**1.34**	**0.08**	**30.20**	**0.21**	**4.69**	**-**	**36.53**
Yellow lupin										
Dukat	0.30	2.46	2.76	3.0	0.3	151.9	n.d.	12.4	0.1	167.6
Mister-PT	0.23	1.31	1.54	4.3	1.1	224.4	2.3	25.9	0.3	258.2
Nacional	0.25	1.44	1.69	3.2	0.2	293.3	n.d.	19.2	0.1	316.0
Taper	0.17	1.49	1.67	5.3	0.4	200.3	n.d.	14.2	0.1	220.3
Mean	**0.24**	**1.68**	**1.91**	**3.9**	**0.5**	**217.5**	**-**	**17.9**	**0.1**	**240.4**
SD	**0.047**	**0.458**	**0.494**	**0.93**	**0.33**	**51.18**	**-**	**5.42**	**0.08**	**54.28**
Chickling vetch										
Grão-da-gramicha	0.44	n.d.	0.44	4.0	0.3	106.7	2.2	21.3	n.d.	134.4
Common vetch										
Barril	0.16	n.d.	0.16	3.0	0.5	23.4	10.1	18.3	0.1	55.3

[1]Results expressed as mean values (n=2). [2]Mean values of Kabuli type varieties (all except var. Elmo). [3]SD; standard deviation. [4]n.d., not detected.

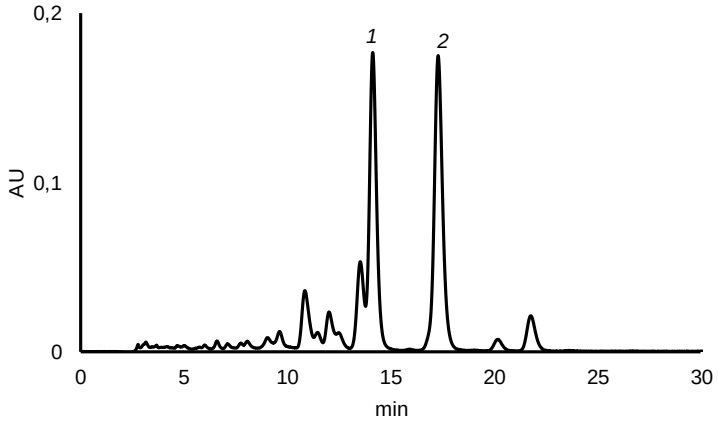

Figure 2. **HPLC-DAD carotenoids profile of chickpea var. Elvar. Peaks identification: lutein (1) and zeaxanthin (2).**

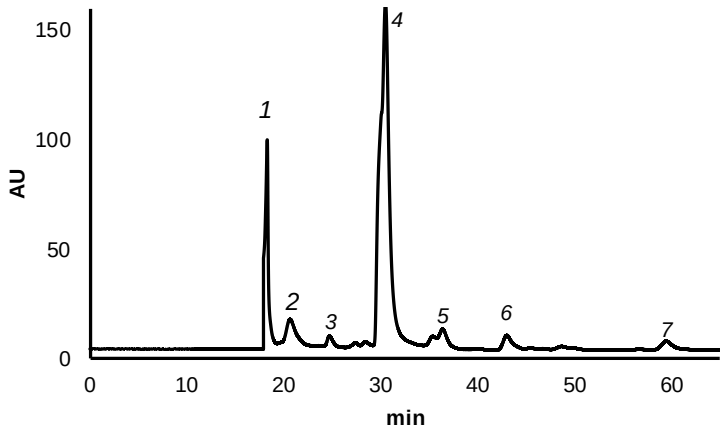

Figure 3. **HPLC-UV organic acids profile of chickpea var. Elvar. Peaks identification: mobile phase (1), oxalic acid (2), *cis*-aconitic acid (2), citric acid (4), malic acid (5), *trans*-aconitic acid (6) and fumaric acid (7).**

3.4. Discriminant analysis

For chickpea, G2 presented, in comparison to G1, higher ($P < 0.05$) levels of C18:1*c*9 and MUFA and lower ($P < 0.05$) levels of C18:2*n*6 and thus of PUFA. C18:3*n*3 tended ($P < 0.1$) to be higher in G1 than in G2. For field pea, G2 tended ($P < 0.1$) to present higher contents than G1 of ADL and C12:0 and showed significantly lower ($P < 0.01$) levels of starch than G1. For the group comprising faba beans, common vetch and chickling vetch, G1 presented significantly higher levels of starch ($P < 0.01$) and C23:0 ($P < 0.05$) and lower ones of lipids and C20:1*c*11 ($P < 0.05$). Moreover, G1 tended ($P < 0.1$) to present higher levels of NDF, C16:0 and C24:0 and lower values of GE and soluble CP than G2. Finally, regarding lupins, G1 showed in relation to G2 significantly ($P < 0.05$) higher ash levels and tended ($P < 0.1$) to present higher contents of total FA and C16:0. However, G2 significantly differed from G1 in individual FA namely C16:1 and C20:0 ($P < 0.05$) and C22:0 and C22:2*n*6 ($P < 0.01$) for which levels were higher.

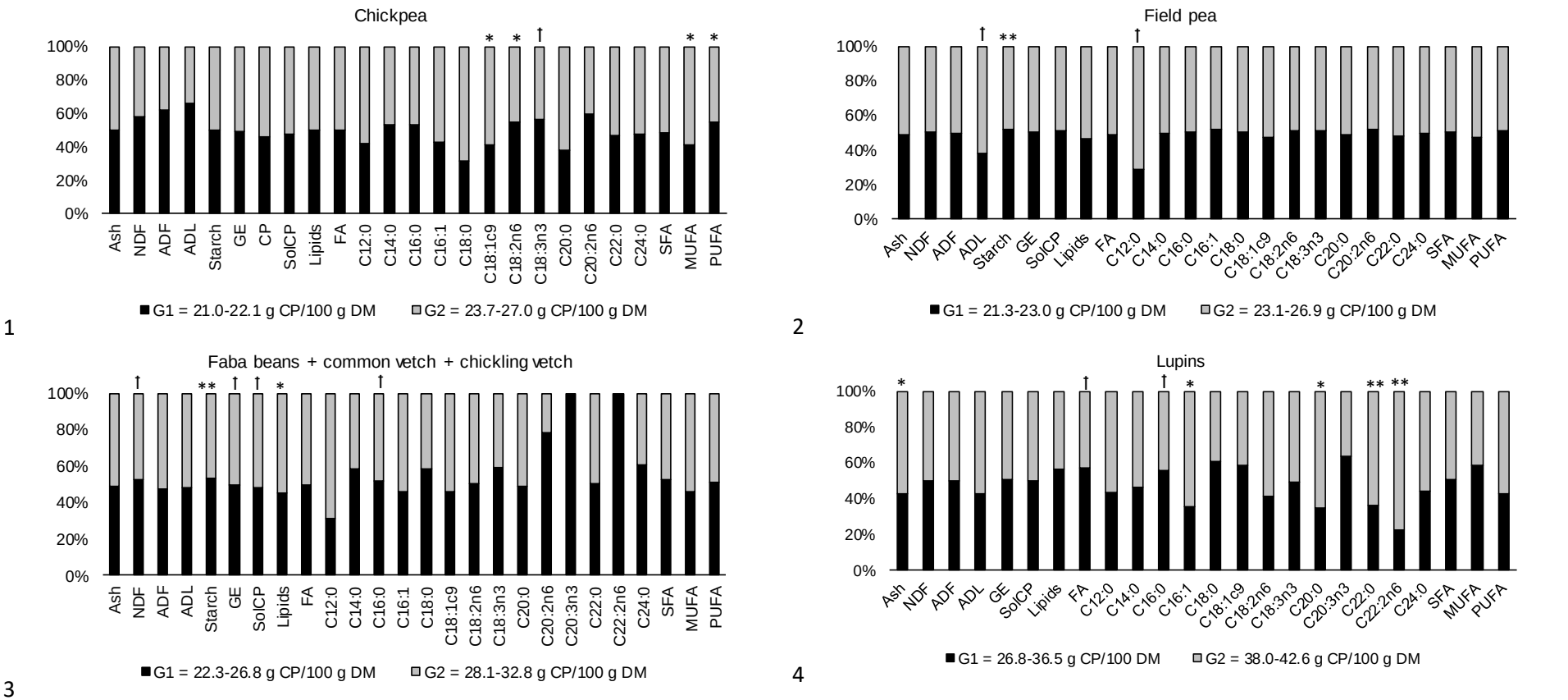

Figure 4. **Analysis of variance (per grain legume group) between groups that represent different crude protein content ranges. NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; GE, gross energy; SolCP, soluble crude protein; FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DM, dry matter. **, *P* < 0.01; *, *P* < 0.05; †, *P* < 0.1; absence of statistical symbol in columns indicate groups are not significantly different (*P* > 0.05).**

4. Discussion

The EU is characterized by a low level of vegetal protein production [24]. Despite the fact the import of large quantities of soybeans and SBM has enabled self-sufficiency in livestock products, increasing the cultivation of GL would be an important contribution to the sustainable development of European agriculture and food systems [25]. Data on the nutritive value of locally-produced GL seeds is therefore useful to promote these alternative ingredients in animal feeding. Moreover, knowledge on the seeds non-nutrients (or secondary compounds) is crucial to avoid toxic events on animals (the case of antinutritional factors) and to take into consideration the benefits these metabolites may bring to farmed animals in terms of, for instance, welfare, zootechnical performances or final product color.

Proximate composition revealed lupins varieties as better sources of CP and of cell-wall components than the other GL, though the poorest sources of starch. Mean CP values found for lupins and other legume seeds are in accordance with known feed tables and other studies [4, 7, 26-28]. However, they may vary slightly depending on variety, maturity and growing conditions [29]. For example, when compared to the CP levels observed in the present work for lupins, Musco *et al.* [27] reported lower ones for the YL varieties Dukat, Mister e Taper (34.3, 36.2 e 32.2 g/100 g DM, respectively) and a higher one for WL var. Multitalia grown in Italy (45.4 g/100 g DM). Still, CP content of all GL studied is quite lower than that of SBM (ca. 50.0 g/100 g DM) and fishmeal (ca. 75 g/100 g DM) [28]. In addition to nutritional properties, GL proteins exhibit functional properties such as solubility, foaming, water and fat binding capacity that play an important role in food formulation and processing. Those properties are determined by the amino acidic composition, structure and conformation as well as processing conditions [30]. About half of seeds CP was highly soluble in water (Table 3) reflecting the presence of water-soluble storage proteins (which may include protease inhibitors and lectins) [29]. Approximately 50% of solubility was previously reported for a chickpea type Kabuli protein isolate after an aqueous alkaline extraction followed by isoelectric precipitation [30].

When compared to other GL, lupins have, indeed, unique carbohydrate properties characterized by high levels of fibre and negligible amounts of starch [7]. Still, energy provided by these seeds is high (Table 3). Seeds of NLL contained more NDF and ADF together than YL and WL, meeting Gdala [31] who also explained that differences may be on the distinct seed content in rhamnose, xylose, galactose, and uronic acids [31]. High concentration of fibre in lupins is considered a drawback mainly in piglets and growing pigs [7, 32] but is interesting for ruminant animals once they well utilize cell-wall components in their diets. Dixon and Hosking [6] reported that fibre from lupin seeds is highly digestible and could favour a good acetate:proprionate ratio in the rumen. In the remaining GL, high starch

levels were observed, as expected [4, 7, 28]. Common vetch (var. Barril), as some FP varieties, achieved more than 40.0 g starch/100 g DM, being in accordance with findings of Abreu and Bruno-Soares [33]. Starch levels above 40.0 g/100 g DM are frequently reported also for FB [7, 28, 33]; in the present work, maximum value was 37.4 g/100g DM (Chiaro di Torrelama). Differences may be attributed to varieties themselves or method of analysis.

Grain legumes contained low amounts of fat with beneficial composition in terms of unsaturated FA, mainly of C18:2*n*6 and C18:1*c*9 which accounted for ca. 60-80% of total FA. C18:1*c*9 was at higher proportion than C18:2*n*6 only in WL samples contributing to their increased MUFA levels and low *n*6/*n*3 ratio, whereas C18:2*n*6 prevailed in all the other GL studied supporting their richness in PUFA. The profile obtained agrees with the literature for most GL species [4, 7, 27, 34]. Little information is available for common vetch; the commercial cultivar “Lanjian NO.3” from China showed different FA profile to var. Barril from Portugal mainly for PUFA as it presented in average 40.0 g C18:3*n*3/100 g FA and only 18.0 g C18:2*n*6/100 g FA [35]. FP and WL varieties approached for C18:3*n*3 similar levels to those of soybean oil (ca. 7.8 g/100 g FA) [35].

Among chickpea varieties, differences were noticed between Desi and Kabuli seeds. Despite the fact only one Desi variety was available for analysis, results suggest that these seeds, more likely to be used in animal feeding, are, comparing to Kabuli ones, richer in cell-wall components and poorer in starch and lipids (still, with higher PUFA proportion). Bampidis and Christodoulou [4] also reported such differences.

Xanthophylls are a class of carotenoids widely used as feed additives to generate products meeting consumers’ demands mainly in terms of color. The application of xanthophylls in animal feed in the EU is restricted to farming of poultry and fish (mainly salmon and trout) [36], lutein being of greatest importance together with astaxanthin and canthaxanthin [37]. When as feed additives, and according to current legislation [36], lutein and zeaxanthin, the two xanthophylls found in the studied GL (Table 4), are only allowed to be fed to poultry. Chickpeas and lupins, while containing the highest levels of zeaxanthin and therefore of total xanthophylls (Table 5), could hence play a major role as sources of natural pigments in poultry diets helping to generate better color of broiler skin and especially of egg yolk [37]. Interestingly, in humans, positive correlations between consumption of lutein/zeaxanthin and adult macula degeneration were ascertained [37]. Greater amounts of carotenoids found in chickpeas and lupins may be associated with their also higher lipid content (Table 4) once carotenoids are fat-soluble compounds. Higher carotenoid levels in chickpea type Desi than in type Kabuli ones can be related to the higher antioxidant activity observed for darker seeds [38] and for which carotenoids may give a contribution.

Among GL, WL varieties presented the highest levels of total organic acids given their increased contents in citric acid (Table 4). Dinkelaker *et al.* [39] showed that, as a way of

mobilizing nutrients (in particular phosphorus) in calcareous soils, white lupins enhanced the release of organic acids such as citric acid from particular root zones. According to these authors, as most of the other lupin species prefer acidic or neutral soils, they do not have to make this effort to acquire nutrients from the soil. This could be the reason why higher levels of citric acid were found in WL seeds comparing to the other GL samples. As natural sources of organic acids, all the studied GL could play a role in animal diets of swine, poultry or fish. Indeed, it is believed that after the ban on most of the antibiotic growth promoters within the EU in 1999, feed additives such as organic acids and probiotics have increased in importance in animal nutrition [40]. In the diets for broilers, acidifiers such as organic acids avoid scouring, maintain the health of the gastrointestinal tract and therefore improve overall zootechnical performances [41]. Used as a supplement for acidification in the diets of rainbow trout, red seabream and rohu, citric acid has been extensively used to enhance growth and feed utilization [42]. Among all organic acids, oxalic acid should be highlighted once it affects calcium and magnesium metabolism and protein digestion when ingested mainly by monogastrics [43]. Where detected (Table 5), this organic acid stood below the levels found for soybean seeds [44].

Discriminant analysis builds a predictive model for group membership. This analysis revealed that more inter-varietal differences occur in the case of lupin seeds. This is probably because, despite of the same genus, the three lupin species (WL, NLL, YL; herein clustered together) differ among each other particularly for FA profile. Within the studied chickpeas, discriminant analysis showed that increased MUFA and decreased PUFA contents (in particular of C18:1*c*9 and C18:2*n*6, respectively) are achieved in seeds with more than 23.6 g CP/100 DM. Both for FP and FB/CoV/CV groups of varieties analyzed, more starch levels are found in seeds with lower CP contents (< 23.1 and 28.1 g CP/100 g DM, respectively). This analysis allows to realize that the choice for a given variety only based on its high CP content may penalize the supply of starch in the case of FP and FP/CoV/CV, PUFA in the case of chickpeas and total FA in the case of *Lupinus* seeds.

5. Conclusions

A fully characterization of the proximate composition, total lipid content, and fatty acids, carotenoids and organic acids profiles of several European marketable GL varieties is herein presented, comprising a valuable tool for those dealing with animal nutrition. Despite lower than that of SBM, protein content of the studied raw mature GL seeds was good (in average 22-40 g/100 g DM). Additionally, high energy levels can also be provided by these seeds

from their starch (in all GL except lupins) and fibre (mainly lupins) fractions, which can strongly dictate which seeds best fit the animal to be fed (e.g. ruminants-fibre issue). All GL varieties revealed to be good sources of unsaturated FA, with C18:1*c*9 and C18:2*n*6 comprising over 60% of total FA.

Chickpeas and lupins presented the highest levels of total carotenoids (xanthophylls), thus suggesting to be putative alternative natural pigments for poultry diets. All varieties but mostly WL varieties could function as natural dietary acidifiers given the levels of organic acids (mainly citric acid) found in the seeds.

Acknowledgements

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Table S1. Fatty acids profile of grain legume samples (g/100 g total fatty acids).

Samples	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1*c*9	C18:2*n*6	C18:3*n*3	C20:0	C20:2*n*6	C20:3*n*3	C22:0	C22:2*n*6	C24:0	NID[1]
Chickpea															
Eldorado	0.01	0.16	11.04	0.03	1.59	24.04	57.00	2.42	0.59	0.06	n.d.[2]	0.33	n.d.	0.21	2.52
Elixir	0.01	0.16	10.70	0.03	1.46	28.63	52.71	2.38	0.56	0.05	n.d.	0.33	n.d.	0.20	2.77
Elmo	0.01	0.21	12.59	0.03	1.38	19.36	59.23	2.89	0.62	0.09	n.d.	0.43	n.d.	0.22	2.96
Elvar	0.01	0.16	10.81	0.02	1.55	25.46	55.47	2.50	0.59	0.06	n.d.	0.33	n.d.	0.20	2.84
Reale	0.01	0.16	9.67	0.04	7.11	34.30	42.83	1.75	1.78	0.03	n.d.	0.53	n.d.	0.30	1.48
Sultano	0.01	0.14	10.32	0.04	1.39	35.49	46.80	1.86	0.60	0.06	n.d.	0.38	n.d.	0.20	2.71
Mean[1]	0.01	0.16	10.51	0.03	2.62	29.58	50.96	2.18	0.82	0.05	-	0.38	-	0.22	2.46
SD[2]	0.000	0.008	0.479	0.007	2.246	4.599	5.353	0.312	0.478	0.012	-	0.077	-	0.039	0.503
Field pea															
Cartouche	0.01	0.23	13.20	0.01	4.01	25.73	45.52	8.55	0.49	0.04	n.d.	0.16	n.d.	0.31	1.73
Cherokee	0.01	0.23	14.38	0.02	2.88	22.38	47.05	10.23	0.47	0.05	n.d.	0.17	n.d.	0.23	1.89
Cigal	0.10	0.20	14.17	0.02	4.24	25.73	42.60	9.98	0.63	0.05	n.d.	0.17	n.d.	0.29	1.79
Comanche	0.01	0.18	14.53	0.02	3.54	16.98	53.05	9.05	0.45	0.06	n.d.	0.16	n.d.	0.29	1.66
Dove	0.01	0.21	13.45	0.03	4.08	24.53	47.31	6.96	0.50	0.04	n.d.	0.16	n.d.	0.35	2.35
Eiffel	0.01	0.17	12.90	0.02	3.59	26.61	43.82	10.22	0.48	0.06	n.d.	0.14	n.d.	0.29	1.69
Enduro	0.01	0.21	15.15	0.02	3.05	18.50	52.82	7.91	0.44	0.07	n.d.	0.16	n.d.	0.31	1.35
Esmeralda	0.03	0.24	14.65	0.04	3.25	19.98	49.56	9.08	0.53	0.06	n.d.	0.19	n.d.	0.31	2.08
Genial	0.01	0.21	13.34	0.04	4.86	19.27	51.65	7.60	0.69	0.06	n.d.	0.18	n.d.	0.29	1.81
Grisel	0.09	0.21	13.16	0.02	3.05	23.66	49.32	7.26	0.46	0.05	n.d.	0.17	n.d.	0.30	2.25
Indiana	0.01	0.25	15.28	0.03	3.16	24.33	44.13	9.90	0.49	0.06	n.d.	0.15	n.d.	0.24	1.98
Isard	0.03	0.16	13.29	0.03	3.20	21.42	50.62	8.62	0.39	0.06	n.d.	0.14	n.d.	0.30	1.73
James	0.02	0.23	14.93	0.03	3.14	15.49	53.02	9.76	0.55	0.09	n.d.	0.18	n.d.	0.23	2.31
Marqueta	0.01	0.33	14.45	0.03	3.79	21.35	49.22	7.69	0.57	0.07	n.d.	0.23	n.d.	0.33	1.93
Montrebei	0.01	0.22	14.91	0.03	4.04	20.92	49.13	7.88	0.53	0.05	n.d.	0.20	n.d.	0.38	1.70

Montsant	0.02	0.28	14.41	0.03	3.82	20.55	50.10	7.61	0.55	0.06	n.d.	0.21	n.d.	0.33	2.03
Pixel-I	0.06	0.14	12.44	0.02	3.07	26.60	46.26	8.46	0.44	0.04	n.d.	0.14	n.d.	0.21	2.09
Pixel-L	0.01	0.16	13.97	0.04	3.82	37.01	36.18	5.84	0.62	0.04	n.d.	0.18	n.d.	0.27	1.87
Spacial	0.01	0.18	12.57	0.03	4.02	19.35	53.00	7.88	0.51	0.06	n.d.	0.15	n.d.	0.26	1.98
Standal	0.02	0.20	13.07	0.03	4.26	28.95	42.92	7.24	0.52	0.04	n.d.	0.16	n.d.	0.28	2.31
Verbal	0.02	0.18	13.29	0.02	3.52	27.69	45.03	7.43	0.39	0.04	n.d.	0.14	n.d.	0.27	1.98
Mean	0.02	0.21	13.88	0.03	3.64	23.19	47.73	8.34	0.51	0.05	-	0.17	-	0.29	1.93
SD	0.026	0.042	0.852	0.008	0.507	4.698	4.228	1.173	0.074	0.013	-	0.024	-	0.040	0.246
Faba bean															
Chiaro di Torrelama	0.03	0.28	16.19	0.03	2.32	21.79	52.12	3.21	1.19	0.09	n.d.	0.48	n.d.	0.30	1.97
Diva	0.02	0.16	13.68	0.02	2.40	24.86	52.38	2.61	1.06	0.10	n.d.	0.47	n.d.	0.24	1.99
Fabelle	0.02	0.12	13.55	0.03	1.86	21.04	56.34	3.05	1.16	0.14	n.d.	0.40	n.d.	0.21	2.08
Favel	0.02	0.33	15.60	0.04	2.53	23.52	50.37	3.26	1.34	0.08	n.d.	0.53	n.d.	0.35	2.03
Gladice	0.02	0.22	14.96	0.04	2.71	26.89	47.71	2.71	1.36	0.10	n.d.	0.51	n.d.	0.26	2.53
Irena	0.18	0.26	14.34	0.03	2.06	25.61	50.62	3.09	1.04	0.12	n.d.	0.43	n.d.	0.27	1.97
Nordica	0.01	0.14	14.76	0.03	1.96	23.98	52.64	2.55	1.02	0.11	n.d.	0.50	n.d.	0.28	2.01
Organdi	0.02	0.24	15.44	0.05	2.77	24.45	49.20	3.01	1.37	0.09	n.d.	0.51	n.d.	0.25	2.59
Rumbo	0.02	0.20	15.11	0.03	2.42	25.91	49.65	2.89	1.14	0.09	n.d.	0.47	n.d.	0.28	1.77
Scuro di Torrelama	0.03	0.25	15.68	0.03	2.18	22.13	52.62	2.70	1.15	0.09	n.d.	0.48	n.d.	0.29	2.37
Mean	0.04	0.22	14.93	0.03	2.32	24.02	51.37	2.91	1.18	0.10	-	0.48	-	0.27	2.13
SD	0.048	0.062	0.821	0.008	0.290	1.814	2.282	0.241	0.125	0.017	-	0.037	-	0.036	0.256
White lupin															
Amiga	0.01	0.09	7.67	0.04	1.49	51.01	18.56	8.77	0.96	0.21	0.05	2.85	0.04	0.58	7.65
Estoril	0.02	0.11	8.35	0.04	1.66	47.79	19.13	7.67	0.89	0.31	0.03	3.21	0.12	0.81	9.89
Lumen	0.02	0.11	8.88	0.07	1.89	55.73	13.56	7.22	0.99	0.13	0.03	3.06	0.03	0.86	7.40
Multitalia-IT	0.02	0.12	9.09	0.05	1.84	47.95	18.92	7.35	0.96	0.26	0.05	3.27	0.10	0.85	9.19
Multitalia-PT	0.02	0.14	9.86	0.07	1.65	47.64	17.77	8.18	0.91	0.23	0.05	3.53	0.09	1.18	8.67

Mean	0.02	0.11	8.77	0.05	1.71	50.02	17.59	7.84	0.94	0.23	0.04	3.18	0.08	0.86	8.56
SD	0.004	0.016	0.733	0.014	0.144	3.115	2.067	0.572	0.037	0.059	0.010	0.226	0.035	0.191	0.933
Narrow-leafed lupin															
Azuro	0.03	0.22	11.56	0.05	6.23	27.91	44.49	4.97	0.79	0.04	n.d.	1.57	n.d.	0.37	1.57
Sonet	0.02	0.18	10.17	0.04	6.84	33.69	40.25	4.80	0.82	0.03	n.d.	1.56	n.d.	0.33	1.25
Mean	0.03	0.20	10.87	0.05	6.54	30.80	42.37	4.89	0.81	0.04	-	1.57	-	0.35	1.41
SD	0.005	0.020	0.695	0.005	0.305	2.890	2.120	0.085	0.015	0.005	-	0.005	-	0.020	0.160
Yellow lupin															
Dukat	0.02	0.16	6.64	0.09	3.01	21.76	48.24	7.41	2.43	0.23	n.d.	5.19	0.17	0.64	4.00
Mister-PL	0.03	0.16	6.20	0.09	2.66	23.51	48.85	6.56	2.09	0.24	n.d.	4.60	0.17	0.58	4.28
Mister-PT	0.04	0.23	7.18	0.13	2.65	30.41	41.84	4.82	1.78	0.17	n.d.	4.69	0.11	0.79	5.14
Nacional	0.03	0.19	7.06	0.08	2.26	20.42	50.14	6.64	1.72	0.23	n.d.	4.21	0.15	0.60	6.30
Taper	0.02	0.16	6.55	0.09	2.67	21.11	49.89	8.09	1.88	0.22	n.d.	4.62	0.15	0.60	3.96
Mean	0.03	0.18	6.73	0.10	2.65	23.44	47.79	6.70	1.98	0.22	-	4.66	0.15	0.64	4.74
SD	0.007	0.028	0.356	0.017	0.238	3.632	3.055	1.095	0.258	0.025	-	0.313	0.022	0.077	0.890
Chickling vetch															
Grão-da-gramicha	0.02	0.63	16.67	n.d.	6.75	11.97	50.80	6.74	1.05	1.85	0.09	0.46	0.22	0.46	2.3
Common vetch															
Barril	0.03	0.19	15.48	0.04	3.47	15.49	54.32	6.11	1.08	0.08	n.d.	0.39	n.d.	0.68	2.65

[1] NID, non-identified fatty acids.
[2] n.d., not detected

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CHAPTER 4: EUROPEAN MARKETABLE GRAIN LEGUME SEEDS: FURTHER INSIGHT INTO PHENOLIC COMPOUNDS PROFILES

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European marketable grain legume seeds: Further insight into phenolic compounds profiles

Sara C.Q. Magalhães [a], Marcos Taveira [b], Ana R.J. Cabrita [a], António J.M. Fonseca [a], Patrícia Valentão [b], Paula B. Andrade [b,*]

[a] REQUIMTE/LAQV, ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal
[b] REQUIMTE/LAQV, Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, R. Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal

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ABSTRACT

Twenty-nine mature raw varieties of grain legume seeds (chickpeas, field peas, faba beans, common vetch and lupins) produced in Europe were investigated for their phenolic profile by means of high performance liquid chromatography coupled to diode array detection (HPLC-DAD). To the best of our knowledge, this study reported for the first time the phenolic composition of mature raw seeds of chickpea type Desi, field pea and common vetch. Phenolic acids were predominant compounds in chickpeas, field peas and common vetch compared to flavonoids, whereas the opposite was observed for lupin seeds. Yellow lupins presented the highest levels of total phenolic compounds followed by narrow-leafed lupins (in average 960 and 679 mg/kg, dry basis, respectively), whereas Kabuli chickpeas got the lowest ones (in average 47 mg/kg, dry basis). Principal component analysis revealed that flavones and total levels of phenolic compounds were responsible for nearly 51% of total data variability.

1. Introduction

Grain legume seeds (Fabaceae) are used for food and feed purposes, constituting excellent sources of proteins, vitamins, minerals, fibers and polyunsaturated fatty acids (Bouchenak & Lamri-Senhadji, 2013). Besides the nutritional value, they also contain non-nutrients or bioactive compounds, such as inhibitors of proteases and amylases, lectins, saponins, phytic acid and phenolic compounds. Phenolic compounds are of particular interest because, besides contributing to the seed color and sensory characteristics of the seed, they provide several biological properties to these ingredients that include anti-allergenic, anti-artherogenic, anti-inflammatory, antimicrobial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Balasundram, Sundram, & Samman, 2006). The main groups of phenolic compounds found in grain legumes are phenolic acids, flavonoids and condensed tannins, which mostly occur as conjugates with mono-, di- and oligosaccharides, linked to one or more of the phenolic groups, and as functional derivatives, such as esters and methyl esters (Balasundram et al., 2006).

Among grain legumes, scarce data is, in general, available on the phenolic compounds profiling of mature raw varieties of chickpea (*Cicer arietinum* L.), field pea (*Pisum sativum* L. (Partim.)), faba bean

* Corresponding author.
E-mail address: pandrade@ff.up.pt (P.B. Andrade).

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(*Vicia faba* L. var. minor), common vetch (*Vicia sativa* L.) and white, narrow-leafed and yellow lupins (*Lupinus albus* L., *L. angustifolius* L. and *L. luteus* L., respectively). For example, chickpeas with dark seed coat, so called of Desi type, are known for their higher antioxidant activity, arising from the phenolics fraction, in comparison to cream to beige seeds (Kabuli type; Segev et al., 2011, 2010). However, as far as we are aware of, the individual phenolics of Desi chickpeas have never been described before. Regarding *P. sativum* and *V. faba*, detailed studies on this topic mainly concern to immature seeds (vegetables), so called peas (Dueñas, Estrella, & Hernández, 2004; Troszynska & Ciska, 2002; Troszynska, Estrella, López-Amóres, & Hernández, 2002) and broad beans (var. major; Abu-Reidah, del Mar Contreras, Arraez-Roman, Fernandez-Gutierrez, & Segura-Carretero, 2014; Baginsky et al., 2013; Nozzolillo, Ricciardi, & Lattanzio, 1989), respectively, as this corresponds to the main form of consumption by humans. Nevertheless, scarce data is available for varieties of these two species, which are intended to be harvested as mature seeds (so called field peas and faba beans, respectively) and whose main target is the animal feed industry. Indeed, field peas and faba beans constitute excellent protein sources for farmed animals, being inclusively increasing in interest as potential substitutes of high priced ingredients (e.g. soybean meal; Jezierny, Mosenthin, & Bauer, 2010). Common vetch seeds were reported to contain the highest polyphenols content and the strongest antioxidant activity among *Vicia* species (Pastor-Cavada et al., 2011) and also to present three and five times more polyphenols and flavonoids than soybean seeds, respectively (Megías et al., 2009). However, their individual phenolic compounds composition was not studied in depth yet. Regarding mature raw seeds of lupin species, more information is available (Dueñas, Hernández, Estrella, & Fernández, 2009; Siger et al., 2012), which is probably explained by the increased interest in these seeds for food and feed purposes (as flours; Pilegaard & Gry, 2009; Villarino, Jayasena, Coorey, Bell, & Johnson, 2015).

It is frequently referred that genotype has primary influence on the content of phenolic compounds in grain legume seeds (Baginsky et al., 2013; El-Mergawi & Taie, 2014; Talukdar, 2013; Wang et al., 1998) followed by the degree of maturity and environmental conditions (Marathe, Rajalakshmi, Jamdar, & Sharma, 2011). Thus, given the importance of screening different genotypes, the present work aimed at investigating the phenolics composition of several mature raw varieties of grain legume species with commercial interest for consumption, to more accurately establish their phytochemical profiles.

2. Materials and methods

2.1. Sampling

Grain legumes studied are part of the European Plant Variety Database (PVD, 2015), being easily marketed throughout the European Union and worldwide. They included mature raw whole seeds of five Kabuli (CHK) and one Desi (CHD) chickpeas, six field peas (FP), six faba beans (FB), four white lupins (WL), two narrow-leafed lupins (NLL), four yellow lupins (YL) and one common vetch (CV). Chickpeas var. Elmo (Desi type), Eldorado, Elixir and Elvar (all Kabuli type), FP var. Grisel and Pixel, FB var. Favel, WL var. Estoril and Mutitalia, NLL var. Azuro, YL var. Mister and Nacional and CV var. Barril were from Portugal; chickpeas var. Reale and Sultano (both of Kabuli type) and FB var. Scuro di torrelama and Chiaro di torrelama were from Italy; FP var. Esmeralda, Montsant, Marqueta and Montrebei were from Spain; FB var. Diva, Fabelle and Organdi and WL var. Amiga and Lumen were from France; finally, NLL var. Sonet and YL var. Dukat and Taper were from Poland.

After reception, seeds were dried in a forced-air oven (65 °C, 24 h) and grounded to 1 mm for analysis. Dry matter (DM) content of grain legume flours was determined after drying the powdered samples at 103 ± 2 °C overnight (AOAC, 2000).

2.2. Standards, reagents and solid-phase extraction columns

Apigenin-6-*C*-glucoside (⩾99.0%), apigenin-7-*O*-neohesperoside (⩾99.0%), apigenin-8-*C*-glucoside (⩾99.0%), (−)-epicatechin (⩾99.0%), gallic acid (⩾99.0%), *p*-hydroxybenzoic acid (⩾90.0%), luteolin-3,7-di-*O*-glucoside (⩾97.0%), luteolin-6-*C*-glucoside (⩾99.0%), luteolin-8-*C*-glucoside (⩾99.0%), myricetin-3-*O*-rhamnoside (⩾99.0%), quercetin-3-*O*-galactoside (⩾98.0%) and quercetin-3-*O*-rhamnoside (⩾98.5%) were purchased from Extrasynthése (Genay, France), whereas gentisic acid (98.0%), syringic acid (>95.0%) and protocatechuic acid (⩾97.0%) were purchased from Sigma Aldrich (St. Louis, MO).

Methanol LiChrosolv was purchased from Merck (Darmstadt, Germany), formic acid (99–100%) from ChemLab (Zedelgem, Belgium) and HCl from VWR Chemicals (Radnor, PA). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA). The C18 non-end-capped columns (50 µm particle size, 60 Å porosity; 10 g of sorbent mass/70 mL of reservoir volume) were obtained from Chromabond (Macherey-Nagel, Germany).

2.3. Phenolics extraction

The extraction procedure was as described by Taveira et al. (2009) with slight modifications. Five grams of dried and milled sample were sonicated for 15 min and agitated (300 rpm) for 30 min at 30 °C with 50 mL of methanol:water (1:1, v/v). The extract was centrifuged (10 min, 4000 rpm) and the vegetal material re-extracted with 50 mL of methanol:water (1:1, v/v) under the same conditions. The combined supernatants were evaporated to dryness under reduced pressure, at 30 °C, and redissolved in 50 mL of water acidified to pH 2 with HCl. The solution obtained was applied in the C18 column, previously conditioned with 70 mL of methanol and 30 mL of acidified water. Polar compounds were collected with the aqueous solvent and the retained phenolic compounds were then eluted with 50 mL of methanol. The purified hydromethanolic extract was concentrated to dryness under vacuum, redissolved in an appropriated volume of methanol LiChrosolv and filtered through membrane filter (pore size 0.45 µm, Millipore, Bedford, MA) for further analysis by high performance liquid chromatography with diode array detection (HPLC-DAD).

2.4. HPLC-DAD analysis

The separation of phenolic compounds was carried out as previously reported (Grosso, Valentão, Andrade, & Andrade, 2015) using an HPLC unit (Gilson) and a 250 × 4.6 mm, i.d., 5 µm Spherisorb ODS2 column (Waters, Milford, MA). The solvent system was a gradient of 5% formic acid in water (A) and methanol (B), starting with 5% B and installing a gradient to obtain 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 45% B at 39 min, 45% at 42 min, 55% B at 47 min, 75% B at 56 min and 100% B at 60 min. Detection was achieved with a Gilson DAD. Spectroscopic data from all peaks were recorded at 280 nm (hydroxybenzoic acids and flavan-3-ols), 320 nm (hydroxycinnamic acids) and 350 nm (flavonoids). The data were processed on Clarity Chromatography station version 6.0 (Data Apex, Prague, Czech Republic). Peak purity was checked by the software contrast facilities.

The identity of the different phenolic compounds was achieved when both their UV–vis spectra, in the 200–400 nm range, and retention time matched with those of pure standards analyzed under the same conditions. Phenolic compounds quantification

was achieved by the absorbance recorded in the chromatograms relative to external standards. Triplicate analyses were performed.

2.4.1. Linearity

The linearity range of the method was assessed by building calibration curves using, at least, six different concentration levels of the pure standards, according to the range of concentrations present in the samples.

2.4.2. Limits of detection and of quantification

Limit of detection (LOD) and limit of quantification (LOQ) of the identified compounds were determined from calibration curve data, following the formula:

$$LOD = (3.3 \times SD)/b$$

$$LOQ = (10 \times SD)/b$$

where SD is the residual standard deviation of the linear regression, and b is the slope of the regression line.

2.5. Statistical analysis

All statistical analyses involving the experimental data were performed using SPSS® 22.0 (IBM, NY, USA). Mean values were compared by one-way analysis of variance (one-way ANOVA). When ANOVA indicated a significant difference ($P < 0.05$), Tukey's HSD *post-hoc* test was performed. Principal component analysis (PCA), based on normalized data, was applied for reducing the number of variables (five variables corresponding to the total phenolic compounds content of grain legumes varieties and to each phenolic compound class: phenolic acids, flavan-3-ols, flavones and flavonols) to a smaller number of the new derived variables (principal components, PCs) that adequately summarize the original information, i.e., the phenolic compounds composition of the studied grain legumes varieties. Phenolic acids included gallic acid, gentisic acid, *p*-hydroxybenzoic acid, protocatechuic acid and syringic acid; flavan-3-ols included epicatechin; flavones included apigenin and luteolin derivatives, while flavonols included myricetin and quercetin derivatives. The PCA method shows similarities between samples projected on a plan and makes it possible to identify which variables determine these similarities and in what way.

3. Results and discussion

In the present work, HPLC-DAD was used to study the phenolic compounds profile of several varieties of marketable grain legume species. Its advantage comparing to spectrophotometric techniques, commonly used for the phenolics study of grain legumes (Marathe et al., 2011; Martinez-Villaluenga et al., 2009; Pastor-Cavada, Juan, Pastor, Alaiz, & Vioque, 2009; Segev et al., 2011; Talukdar, 2013), is the sensitivity of analysis, ideal for both separation and quantification of phenolic compounds (Khoddami, Wilkes, & Roberts, 2013). Indeed, despite being simple, quick and economic, spectrophotometric techniques are not selective and may include phenols bounded to proteins. In addition, they give an estimation of phenolic compounds concentrations above a certain minimum level and do not quantify phenolics individually (Khoddami et al., 2013; Troszynska et al., 2002).

The calibration plots of all the phenolic compounds identified by HPLC-DAD (Supplementary material 1 and 2) exhibited correlation coefficient values higher than 0.99 (Table 1). Their calculated LOD and LOQ values are also shown in Table 1.

3.1. Chickpeas

Table 2 presents the phenolics composition of the chickpea extracts analyzed. All chickpea varieties presented in common *p*-hydroxybenzoic and gentisic acids, whereas syringic acid was found only in the dark variety (Elmo). Other major difference found between Desi and Kabuli chickpeas concerned to the presence, in the former, of flavonoids comprising glycosides of luteolin, myricetin and quercetin. Although no flavonoid was detected herein in Kabuli varieties, such compounds were reported before in these seeds and comprised derivatives of pinocembrin, quercetin, kaempferol and biochanin (Aguilera et al., 2011). To the best of our knowledge, the phenolics profile of Desi chickpeas was herein characterized for the first time.

Kalogeropoulos et al. (2010) reported equal contents of both phenolic acids and flavonoids fractions in the seeds of soaked and cooked chickpea. In the present work, phenolic acids predominated over flavonoids in all raw chickpeas. *p*-Hydroxybenzoic acid was the major phenolic acid in Kabuli varieties (ca. 19–61 mg/kg DM; Table 2) agreeing with Aguilera et al. (2011); those authors emphasized the importance of this compound in relation to antioxidant, anti-inflammatory and antimicrobial properties.

Total phenolic compounds content was cultivar-dependent ($P < 0.001$), Elmo cultivar presenting the highest value (ca.

Table 1
UV data, linearity, limit of detection (LOD) and limit of quantification (LOQ) of the identified phenolic compounds.

Phenolic compound	λ_{max} (nm)	Regression equation	R^{2a}	Linearity	LOD	LOQ
				mg/mL		
Gallic acid	270	$y = 6.1 \times 10^4x + 1959.8$	1.000	0.105–0.836	0.024	0.074
Protocatechuic acid	259, 292	$y = 3.2 \times 104x + 282.6$	1.000	0.019–0.306	0.001	0.004
p-Hydroxybenzoic acid	255	$y = 3.3 \times 10^4x + 830.8$	0.999	0.098–0.780	0.016	0.050
Gentisic acid	246, 330	$y = 2.8 \times 10^4x + 958.7$	0.998	0.064–1.030	0.029	0.086
Epicatechin	275	$y = 1.7 \times 10^4x + 183.7$	0.998	0.064–0.510	0.018	0.055
Syringic acid	274	$y = 7.3 \times 10^4x + 1344.0$	0.999	0.090–0.720	0.016	0.049
Luteolin-8-*C*-glucoside	257, 266, 292sh, 348	$y = 6.1 \times 10^4x + 157.2$	0.999	0.013–0.100	0.002	0.007
Luteolin-6-*C*-glucoside	257, 269, 348	$y = 7.4 \times 10^4x + 67.6$	0.999	0.007–0.050	0.001	0.004
Apigenin-8-*C*-glucoside	268, 300sh, 337	$y = 5.6 \times 10^4x + 602.6$	0.999	0.098–0.392	0.011	0.033
Luteolin-3,7-di-*O*-glucoside	268, 340	$y = 1.6 \times 10^4x + 52.7$	1.000	0.115–0.460	0.010	0.029
Myricetin-3-*O*-rhamnoside	260, 300sh, 350	$y = 3.7 \times 10^4x + 232.1$	1.000	0.039–0.310	0.006	0.017
Apigenin-6-*C*-glucoside	270, 334	$y = 6.0 \times 10^4x + 128.5$	1.000	0.275–2.200	0.039	0.117
Quercetin-3-*O*-galactoside	257, 268sh, 298sh, 360	$y = 5.0 \times 10^4x + 261.9$	1.000	0.024–0.190	0.002	0.005
Quercetin-3-*O*-rhamnoside	256, 264sh, 301sh, 349	$y = 4.2 \times 10^4x + 525.5$	1.000	0.098–0.390	0.017	0.050
Apigenin-7-*O*-neohesperoside	266, 335	$y = 4.2 \times 10^4x + 225.0$	1.000	0.063–0.250	0.007	0.023

[a] R^2, correlation coefficient.

Table 2
Quantification of phenolic compounds (mg/kg, dry basis) of seeds of chickpea (*Cicer arietinum*).[1]

Compounds	Elmo	Eldorado	Reale	Sultano	Elixir	Elvar
Phenolic acids						
p-Hydroxybenzoic acid	35.1 (2.74)^{a}	37.5 (0.03)^{a}	60.5 (0.53)^{b}	33.7 (0.59)^{a}	19.2 (0.29)^{c}	23.9 (0.21)^{d}
Syringic acid	45.9 (0.45)	nd	nd	nd	nd	nd
Gentisic acid	26.0 (0.47)^{a}	15.3 (0.02)^{b}	18.9 (1.26)^{c}	9.7 (0.03)^{d}	8.3 (0.05)^{d}	8.1 (0.18)^{d}
Σ[2]	107.0	52.8	79.4	43.4	27.5	32.0
Flavonoids						
Luteolin-8-*C*-glucoside	1.2 (0.08)	nd	nd	nd	nd	nd
Myricetin-3-*O*-rhamnoside	7.4 (0.51)	nd	nd	nd	nd	nd
Quercetin-3-*O*-galactoside	7.2 (0.11)	nd	nd	nd	nd	nd
Quercetin-3-*O*-rhamnoside	5.0 (0.01)	nd	nd	nd	nd	nd
Σ[2]	20.8	0.0	0.0	0.0	0.0	0.0
Total phenols	127.8^{a}	52.8^{c}	79.4^{b}	43.4^{d}	27.5^{e}	32.0^{e}

[1] Results expressed as mean (standard deviation) of three determinations; nd, not detected. Different superscript letters in the same row indicate significant differences ($P < 0.001$).
[2] Σ, sum of the identified compounds.

128 mg/kg DM; Table 2). The higher amount of phenolic compounds found in the dark variety agrees with previous works on raw chickpeas (Segev et al., 2011, 2010), being attributed to the seed size, besides to the dark color of the seed coat: the higher proportion of hulls in smaller seeds results in higher amounts of phenolic compounds (Marathe et al., 2011). Consequently, stronger antioxidant activity is usually observed for colored chickpea lines (Segev et al., 2011). Values for total phenolics observed for the Kabuli varieties (ca. 28–79 mg/kg DM; Table 2) approached those of other Kabuli chickpeas (ca. 67–92 mg/kg DM; Aguilera et al., 2011). Kalogeropoulos et al. (2010) found ca. 14 mg of total polyphenols/kg seed (fresh weight) in soaked and cooked chickpeas, having concluded that the process treatment causes partial leaching and thermal/oxidative deterioration of these compounds.

3.2. Field peas

Table 3 shows the phenolics composition of the analyzed FP extracts. Protocatechuic and *p*-hydroxybenzoic acids were found in all of them. These phenolic acids had been previously reported in white and colored pea varieties (Dueñas et al., 2004; Troszynska & Ciska, 2002; Troszynska et al., 2002) and in FP (together with syringic, *trans*-ferulic and *trans*-*p*-coumaric acids; Sosulski & Dabrowski, 1984). This is, to the best of our knowledge, the first report on the overall phenolic compounds profile of FP varieties once the study of Sosulski and Dabrowski (1984) focused solely on the phenolic acids fraction of FP (and of other legume seeds).

Glycosylated flavones, namely luteolin and apigenin derivatives, were the flavonoids detected. While apigenin-8-*C*-glucoside had been previously identified in pea seed coats (Dueñas et al., 2004; Troszynska et al., 2002), apigenin-6-*C*-glucoside, luteolin-6-*C*-glucoside and luteolin-3,7-di-*O*-glucoside are, as far as we are aware of, here reported in whole *P. sativum* seeds for the first time. Flavonols, such as kampferol and quercetin glycosides, were also found in peas (Dueñas et al., 2004; Troszynska et al., 2002). Results from these latter studies showed that flavonols normally occur in pea seeds at quite lower amounts than flavones, which may explain why they were not detected in the studied FP varieties.

Individual and total phenolic compounds content were distinct ($P < 0.001$) between FP varieties. Phenolic acids fraction (ca. 58–248 mg/kg DM) predominated over that of flavonoids (ca. 6–57 mg/kg DM) in all samples. In addition, higher levels of total phenolic acids, and of total phenolic compounds, were found in colored hull seeds (Esmeralda, Montsant, Montrebei) when compared to white to beige hull seeds (Marqueta, Pixel and Grisel), agreeing with the findings of Troszynska and Ciska (2002) with peas. Total amount of phenolic compounds in FP ranged from 97 to 255 mg/kg DM ($P < 0.001$).

3.3. Faba beans

Table 4 presents the phenolics composition of all the FB extracts analyzed. Only one phenolic acid (gallic acid) was identified in all samples. Similarly, Sosulski and Dabrowski (1984) found gallic acid

Table 3
Quantification of phenolic compounds (mg/kg, dry basis) of seeds of field pea (*Pisum sativum* L.).[1]

Compounds	Esmeralda	Montsant	Marqueta	Montrebei	Grisel	Pixel
Phenolic acids						
Protocatechuic acid	67.8 (3.74)^{a}	61.5 (2.40)^{a}	24.6 (1.01)^{b}	163.5 (2.91)^{c}	20.9 (0.05)^{b,d}	12.1 (0.04)^{d}
p-Hydroxybenzoic acid	101.7 (2.39)^{a}	49.8 (5.61)^{b,d}	55.6 (0.60)^{b,d}	84.6 (5.44)^{c}	64.0 (2.76)^{b}	45.4 (1.42)^{d}
Σ[2]	169.5	111.3	80.2	248.2	84.9	57.5
Flavonoids						
Luteolin-6-*C*-glucoside	11.3 (0.33)a	2.4 (0.04)^{b}	1.4 (0.10)^{c}	3.7 (0.11)^{d}	nd	nd
Apigenin-8-*C*-glucoside	21.2 (1.44)^{a}	8.5 (0.06)^{b}	9.0 (0.19)^{b}	nd	nd	nd
Luteolin-3,7-di-*O*-glucoside	nd	nd	nd	nd	15.8 (0.08)^{a}	46.1 (1.73)^{b}
Apigenin-6-*C*-glucoside	24.6 (1.96)^{a}	7.7 (0.03)^{b}	5.9 (0.62)^{b,d}	2.7 (0.14)^{d}	nd	nd
Σ[2]	57.2	18.6	16.4	6.4	15.8	46.1
Total phenols	226.7^{b}	129.9^{c}	96.6^{d}	254.6^{a}	100.7^{d}	103.6^{d}

[1] Results expressed as mean (standard deviation) of three determinations; nd, not detected. Different superscript letters in the same row indicate significant differences ($P < 0.001$).
[2] Σ, sum of the identified compounds.

Table 4
Quantification of phenolic compounds (mg/kg, dry basis) of seeds of faba bean (*Vicia faba* L. var. minor) and common vetch (*Vicia sativa* L.).[1]

Compounds	Faba bean						Common vetch
	Favel	Diva	Fabelle	Organdi	Scuro di torrelama	Chiaro di torrelama	Barril
Phenolic acids							
Gallic acid	22.9 (0.26)^{a}	121.5 (0.51)^{b}	138.2 (1.89)^{c}	110.5 (4.56)^{b}	80.8 (5.93)^{d}	45.8 (0.57)^{e}	nd
Protocatechuic acid	nd	nd	nd	nd	nd	nd	36.9 (0.68)
p-Hydroxybenzoic acid	nd	nd	nd	nd	nd	nd	26.8 (0.33)
Σ[2]	22.9	121.5	138.2	110.5	80.8	45.8	63.7
Flavonoids							
Epicatechin	nd	222.4 (2.55)a	119.0 (2.21)^{b}	128.1 (4.86)^{b}	208.5 (18.7)^{a}	28.3 (0.70)^{c}	nd
Luteolin-8-*C*-glucoside	nd	5.9 (0.07)	nd	nd	nd	nd	nd
Luteolin-6-*C*-glucoside	1.1 (0.03)^{a}	3.6 (0.15)^{b}	5.3 (0.34)^{c}	nd	nd	1.4 (0.03)^{a}	nd
Apigenin-8-*C*-glucoside	nd	5.1 (0.05)	nd	nd	nd	nd	nd
Myricetin-3-*O*-rhamnoside	1.8 (0.04)^{a}	8.1 (0.51)^{b}	13.6 (0.15)^{c}	nd	3.9 (0.51)^{d}	2.2 (0.00)^{a}	nd
Apigenin-7-*O*-neohesperoside	3.0 (0.09)	nd	nd	nd	nd	nd	nd
Σ[2]	5.9	245.2	137.9	128.2	212.4	31.9	0.0
Total phenols	28.8^{e}	366.7^{a}	276.1^{b,c}	238.7^{c}	293.2^{b}	77.7^{d}	63.7

[1] Results expressed as mean (standard deviation) of three determinations; nd, not detected. Different superscript letters in the same row between faba bean samples indicate significant differences ($P < 0.001$).
[2] Σ, sum of the identified compounds.

as the major phenolic acid in the hulls of FB. In broad beans, no (Baginsky et al., 2013) or few (e.g. derivatives of salicylic, tartaric and eucomic acids; Abu-Reidah et al., 2014) phenolic acids were found.

The flavonoids identified included epicatechin (a flavan-3-ol), apigenin and luteolin glycosides (flavones) and myricetin-3-*O*-rhamnoside (flavonol), each variety presenting, in general, different profiles. The presence of myricetin-3-*O*-rhamnoside in all samples except Organdi is consistent with studies reporting myricetin derivatives as main flavonols in FB (El-Mergawi & Taie, 2014; Nozzolillo et al., 1989). According to El-Mergawi and Taie (2014), apigenin and quercetin compounds occur in small quantities in raw FB, which agree with our findings. Quercetin was not even detected in the studied FB either in the free or glycosylated form. On the other hand, luteolin-8-*C*-glucoside and luteolin-6-*C*-glucoside were identified for the first time in *V. faba* seeds. These compounds were not reported before in broad beans (Abu-Reidah et al., 2014; Baginsky et al., 2013) neither in raw Egyptian FB varieties (El-Mergawi & Taie, 2014).

The individual and total amounts of phenolic compounds differed among varieties ($P < 0.001$). With the exception of Favel variety, epicatechin occurred at higher levels than flavones and flavonols together, agreeing with results of Baginsky et al. (2013) with broad beans. Bekkara, Jay, Viricel, and Rome (1998) also reported for one FB cultivar (cv. Alfred) the predominance of catechin derivatives in the seeds coat. In the present work, epicatechin levels were highest for Diva and Scuro di torrelama (ca. 209–222 mg/kg DM) and lowest for Chiaro di torrelama (ca. 28 mg/kg DM). Flavan-3-ols have been reported to exhibit several health beneficial effects by acting as antioxidant, anticarcinogen, cardiopreventive, antimicrobial, anti-viral, and neuro-protective agents (Aron & Kennedy, 2008). In addition, polymers of polyhydroxyflavan-3-ol monomers, this is, condensed tannins, occur at high levels in *V. faba* seeds, being considered major compounds in *Vicia* genus (Abu-Reidah et al., 2014; Boudjou, Oomah, Zaidi, & Hosseinian, 2013; Gulewicz, Martinez-Villaluenga, Kasprowicz-Potocka, & Frias, 2014; Sánchez-Chino, Jiménez-Martínez, Dávila-Ortiz, Álvarez-González, & Madrigal-Bujaidar, 2015). If, on one hand, condensed tannins have historically been considered antinutritional factors for binding food/feed, salivary or endogenous proteins and minerals, further decreasing their bioavailability for absorption (Wang et al., 1998) and diets overall palatability, on the other, given their high degree of polymerization, they are now recognized as having excellent properties (Koleckar et al., 2008). Chromatographic separation of condensed tannins is among the most severe challenges because of their structural diversity and large number of isomeric forms (Abu-Reidah et al., 2014), but judging by the amounts of epicatechin found, FB appear as ingredients with strong health-promoting benefits.

Despite Bekkara et al. (1998) having reported that the phenolic pattern of whole FB seed was mainly defined by phenolic acids (compounds not specified), all FB herein studied, excepting Favel and Chiaro di torrelama, presented higher levels of flavonoids than of phenolic acids. Those two varieties (Favel and Chiaro di torrelama) had the lowest amount of total phenolic compounds (ca. 28 and 78 mg/kg DM, respectively), whereas Diva had the highest (ca. 367 mg/kg DM; $P < 0.001$).

3.4. Common vetch

The phenolic compounds of CV extract are also displayed in Table 4. As far as we are aware of, this is the first report on the phenolics profile of *V. sativa* seeds. Two hydroxybenzoic acids, namely, protocatechuic and *p*-hydroxybenzoic acids, were identified in the seeds of CV and, to the best of our knowledge, for the first time also in this species. Other phenolic acids were identified by HPLC in a whole CV plant extract, and included gallic, vanillic and syringic acids (Saleem et al., 2014).

No flavonoid was identified. In the fresh aerial parts of *V. sativa* ssp. *nigra* var. *nigra*, flavonoids including glycosides of apigenin, kaempferol, luteolin and quercetin, were detected by Gamal-Eldeen, Kawashty, Ibrahim, Shabana, and El-Negoumy (2004).

Total phenolics content of CV was of ca. 64 mg/kg DM, quite lower value than that previously reported by means of spectrophotometric techniques (ca. 21 g of total polyphenols/kg seed flour) (Pastor-Cavada et al., 2011). Lower phenolic compounds concentration in CV seeds than in most of FB (Table 4) disagrees with findings of Pastor-Cavada et al. (2011). Recognized good antioxidant activity of CV seeds together with other biological activities arising from the phenolic compounds fraction (e.g. antiproliferative and antibacterial; Amarowicz, Troszyńska, & Pegg, 2008; Gamal-Eldeen et al., 2004; Megías et al., 2009; Saleem et al., 2014) may be of interest from a functional point of view and for the revalorization of this ancient crop.

3.5. Lupins

Table 5 presents the phenolics composition of the WL, NLL and YL extracts analyzed. Protocatechuic acid (a phenolic acid) was

Table 5
Quantification of phenolic compounds (mg/kg, dry basis) of seeds of white lupin (*Lupinus albus* L.), narrow-leafed lupin (*L. angustifolius* L.) and yellow lupin (*L. luteus* L.).[1]

Compounds	White lupin					Narrow-leafed lupin			Yellow lupin				
	Estoril	Amiga	Lumen	Multitalia	*P* value	Azuro	Sonet	*P* value	Dukat	Mister	Nacional	Taper	*P* value
Phenolic acids													
Protocatechuic acid	nd	nd	7.5 (0.24)	nd	–	nd	nd	–	10.0 (0.23)^a	13.4 (0.78)^b	27.6 (0.11)^c	13.1 (0.00)^b	***
Flavonoids													
Apigenin heteroside[2]	130.3 (1.96)^a	465.2 (0.78)^b	146.0 (0.63)^c	159.5 (1.42)^d	***	665.9 (30.62)	692.0 (2.12)	ns	789.5 (3.14)^a	706.0 (16.17)^a	1137.4 (8.51)^b	1143.7 (60.37)^b	***
Total phenols	130.3^a	465.2^b	153.5^c	159.5^c	***	665.9	692.0	ns	799.5^a	719.4^a	1165.0^b	1156.8^b	**

[1] Results expressed as mean (standard deviation) of three determinations; nd, not detected. Different superscript letters in the same row indicate significant differences: ***, $P < 0.001$; **, $P < 0.01$. ns, not significant.
[2] Tentatively identified compound based on its UV spectra together with data from (Siger et al., 2012) and quantified as apigenin-8-*C*-glucoside.

mainly present in YL. Besides protocatechuic acid, other phenolic acids, such as *p*-hydroxybenzoic and gallic acids, have also been reported in raw lupin species (Dueñas et al., 2009; Siger et al., 2012).

In the chromatograms of WL, NLL and YL varieties, registered at 350 nm, a dominant peak was observed at 21.6 min, presenting an UV spectrum with maxima absorption at 246.8(sh), 271 and 335 nm (Supplementary material 1 and 2), pointing to a flavonoid, in particular to an apigenin derivative. This goes in accordance with results obtained by Siger et al. (2012) with hydromethanol extracts from the same lupin species. Indeed, those authors found two apigenin *C*-glucosides eluting very close to one another, which they further identified by HPLC/MS^n as apigenin-6,8-di-*C*-β-glucopyranoside and apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside. Besides apigenin derivatives, Dueñas et al. (2009) also found luteolin glycosides and diosmetin in raw NLL, concluding that flavones represented more than half of the identified phenolics. Nevertheless, no luteolin derivatives were detected in the samples analyzed herein.

Yellow lupin varieties exhibited the highest phenolic acid and flavonoid contents, agreeing with results of Siger et al. (2012). Mean contents herein obtained for the apigenin heteroside in the three lupin species (YL > NLL > WL) are also in accordance with that study. In addition, the total amount of phenolic compounds significantly varied within WL ($P < 0.001$; ca. 130–465 mg/kg DM) and YL ($P < 0.01$; ca. 719–1165 mg/kg DM) varieties, whereas similar values were found for both NLL varieties ($P > 0.05$; ca. 666–692 mg/kg DM).

3.6. Comparison between groups of grain legumes

Mean total phenolic compounds content (mg/kg DM ± standard deviation) of grain legumes varied in the following decreasing order: YL (ca. 960 ± 203.9) > NLL (ca. 679 ± 20.2) > WL (ca. 227 ± 137.9) > FB (ca. 214 ± 120.6) > FP (ca. 152 ± 64.2) > CHD (ca. 128) > CV (ca. 64) > CHK (ca. 47 ± 18.4). High standard deviation values reflect the intraspecific variation. When studying several Indian grain legumes (white and colored peas, chickpeas, beans, among others), Marathe et al. (2011) also found peas and chickpeas among the seeds with lowest phenolics content and, therefore, with the weakest antioxidant potential.

Having observed distinct composition in terms of total and also individual phenolic compounds, PCA was applied to identify patterns in our dataset that highlight similarities and differences between grain legumes varieties (Fig. 1). Two PCs were retained, corresponding to eigenvalues > 1, and explained 79.05% of total data variability. The first one (PC1) represented 50.88% of the variation and was associated with flavones and total phenolic compounds content, whereas component two (PC2), responsible for 28.17% of the variation, was associated with flavan-3-ols and flavonols. Three groups can be clearly distinguished. One group (G1) includes all NLL and YL varieties and also WL var. Amiga, all placed in the positive plan of PC1 and PC2 for being the samples with the highest levels of flavones and of total phenolic compounds. Group 2 (G2), positioned in the positive plan of PC2 and negative plan of PC1, is constituted by all FB varieties (excepting Favel and Chiaro di torrelama) and by CHD. Those FB are the richest samples in terms of flavan-3-ols (>100 mg/kg DM; Table 4) and together with CHD present the highest levels of flavonols (excepting FB var. Organdi). Finally, group 3 (G3) comprised all CHK and FP varieties and those of FB and WL not included in the aforementioned groups. It contains the varieties with the lowest levels of total phenolic compounds, this is, with less than 255 mg/kg DM. Within G3, a

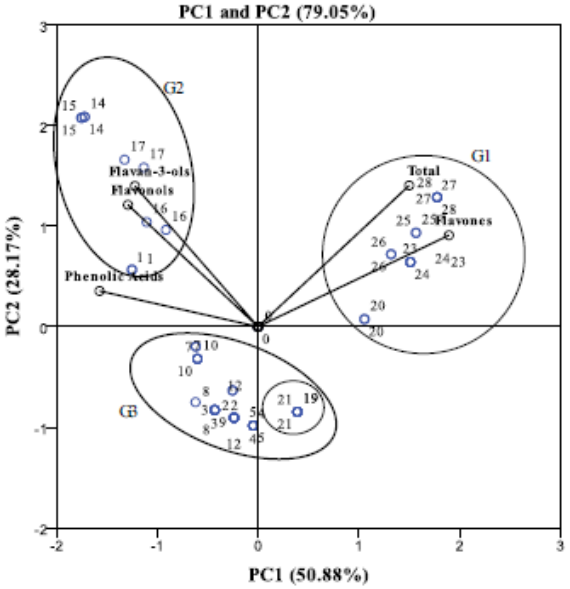

Fig. 1. Projection of grain legumes varieties and loadings by classes of phenolic compounds and total phenolic compounds content into the plane composed by the principal components PC1 and PC2, containing 79.05% of the total variance. 1, Elmo; 2, Eldorado; 3, Reale; 4, Sultano; 5, Elixir; 6, Elvar; 7, Esmeralda; 8, Montsant; 9, Marqueta; 10, Montrebei; 11, Grisel; 12, Pixel; 13, Favel; 14, Diva; 15, Fabelle; 16, Organdi; 17, Scuro di torrelama; 18, Chiaro di torrelama; 19, Estoril; 20, Amiga; 21, Lumen; 22, Multitalia; 23, Azuro; 24, Sonet; 25, Dukat; 26, Mister; 27, Nacional; 28, Taper; 29, Barril.

sub-group formed by the remaining WL varieties can be noticed; its placement in the positive plan of PC1 results from the higher contents in total phenolic compounds and flavones comparing to the other G3 varieties.

4. Conclusions

The HPLC-DAD was successfully applied to characterize the phenolics profile of 29 genotypes of grain legumes produced in Europe. As far as we are aware of, mature raw whole seeds of chickpea type Desi, field pea and common vetch were characterized for the individual phenolics profile for the first time in the present work. Regarding Kabuli chickpeas, faba beans and lupins, a further insight into their phenolics profile was achieved with the characterization of varieties/genotypes not studied to date. Overall, the observed inter- and intraspecific variation, concerning to qualitative and quantitative phenolic profiles of grain legumes, can be useful to provide food/feedstuffs with specific phenolics composition, as well as products with specific health benefits arising from these metabolites.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.07.152.

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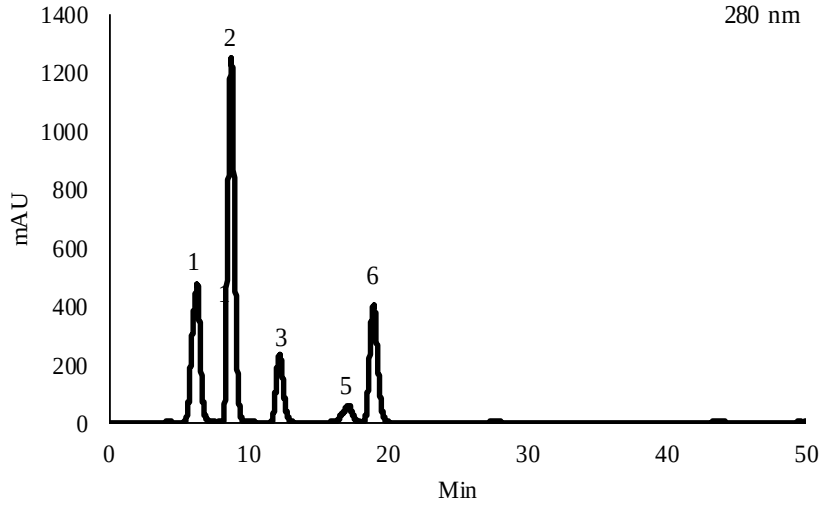
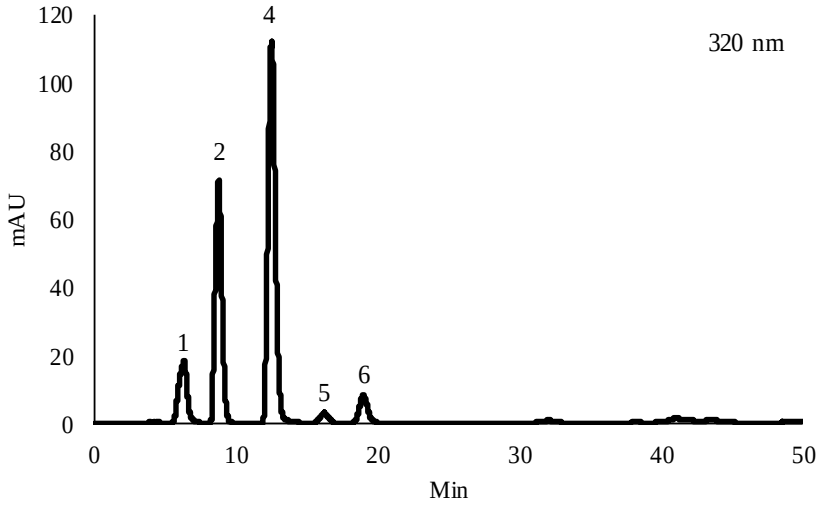
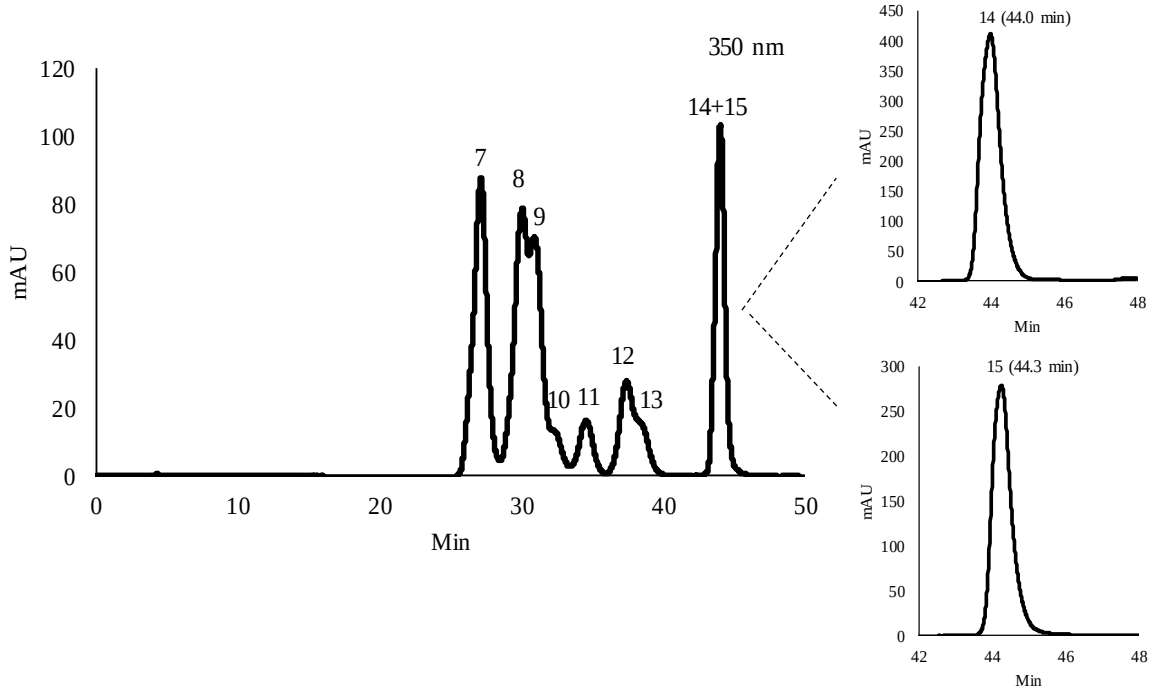
Supplementary material 1. HPLC-DAD representative chromatograms of pure standards and of grain legumes samples. (**1**) gallic acid, (**2**) protocatechuic acid, (**3**) *p*-hydroxybenzoic acid, (**4**) gentisic acid, (**5**) epicatechin, (**6**) syringic acid, (**7**) luteolin-8-*C*-glucoside, (**8**) apigenin-8-*C*-glucoside, (**9**) luteolin-6-*C*-glucoside, (**10**) luteolin-3,7-di-*O*-glucoside, (**11**) myricetin-3-*O*-rhamnoside, (**12**) apigenin-6-*C*-glucoside, (**13**) quercetin-3-*O*-galactoside, (**14**) quercetin-3-*O*-rhamnoside, (**15**) apigenin-7-*O*-neohesperoside, (**a**) apigenin heteroside (from *Lupinus* spp. samples).

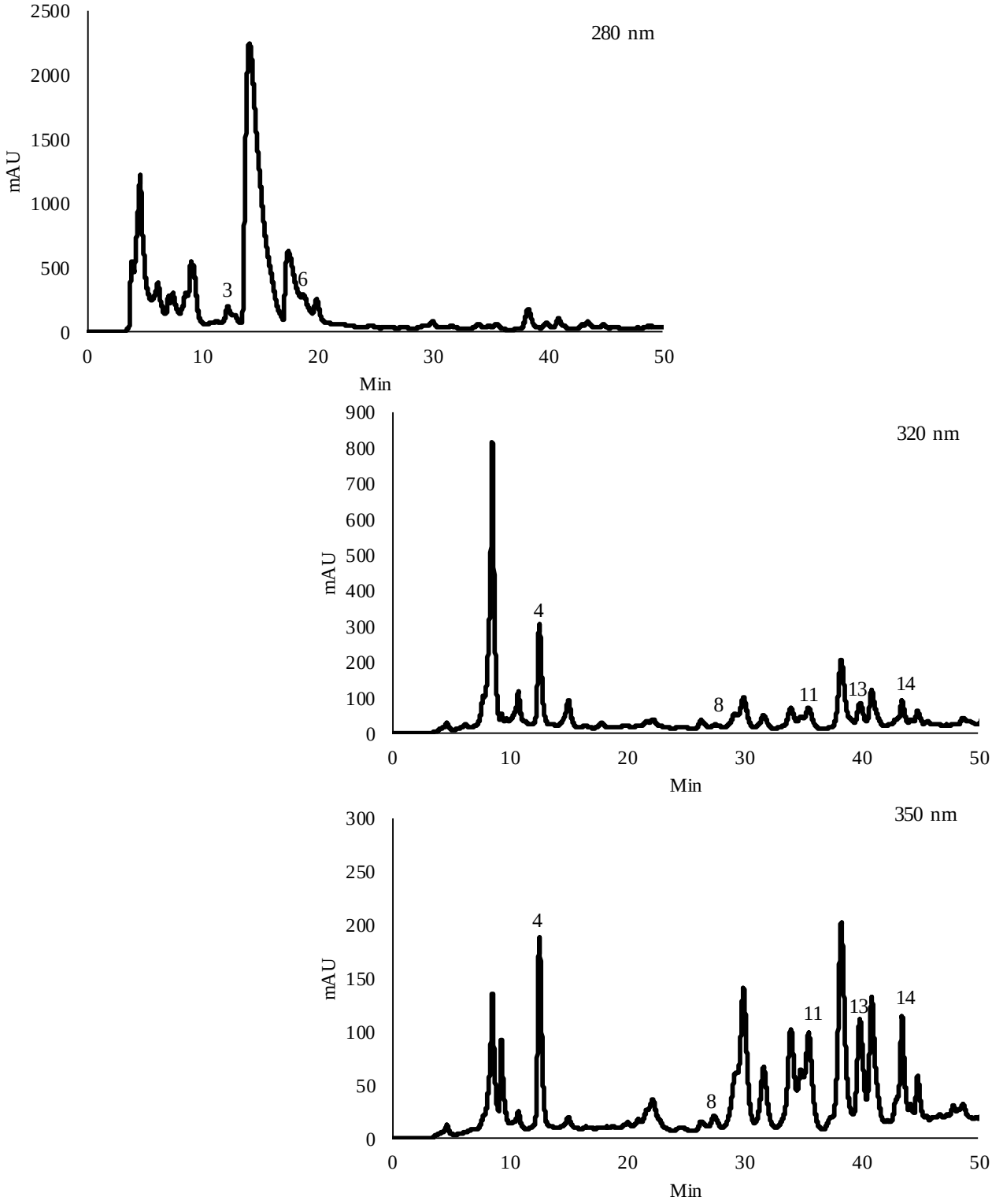
Pure standards



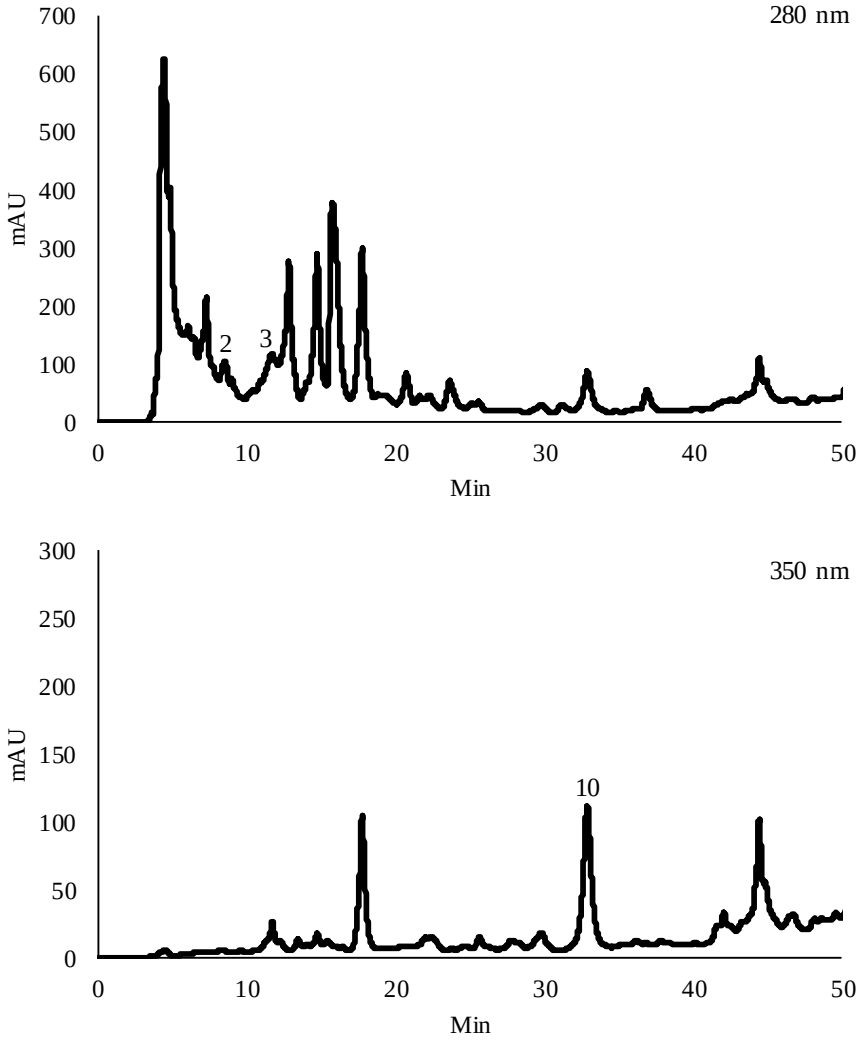




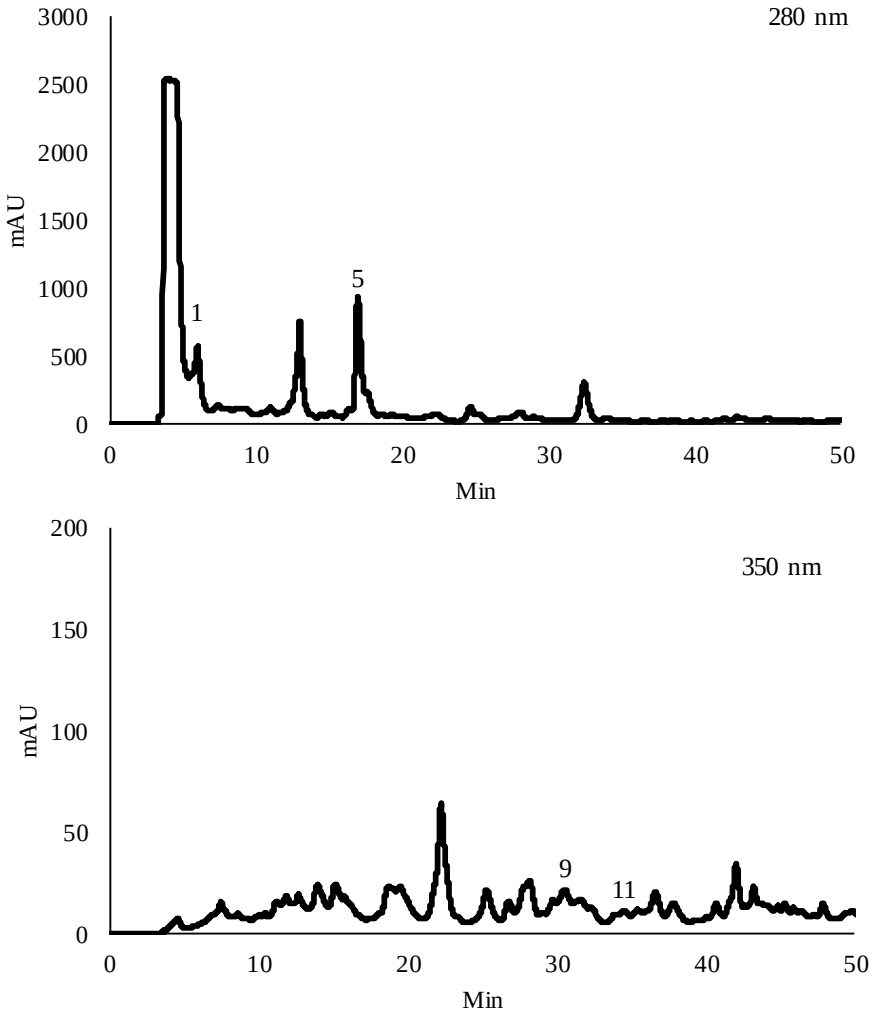
***Cicer arietinum* L. var. Elmo**



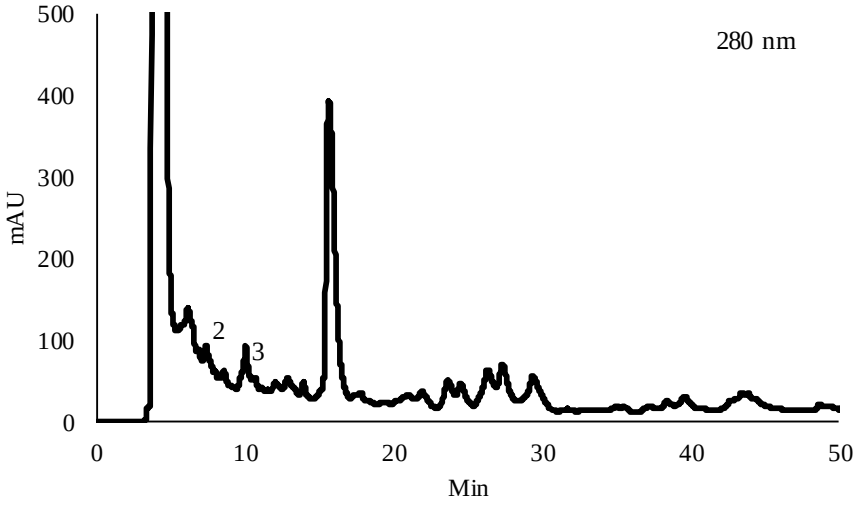
***Pisum sativum* L. var. Pixel**



***Vicia faba* L. var. Chiaro di torrelama**



***Vicia sativa* L. var. Barril**



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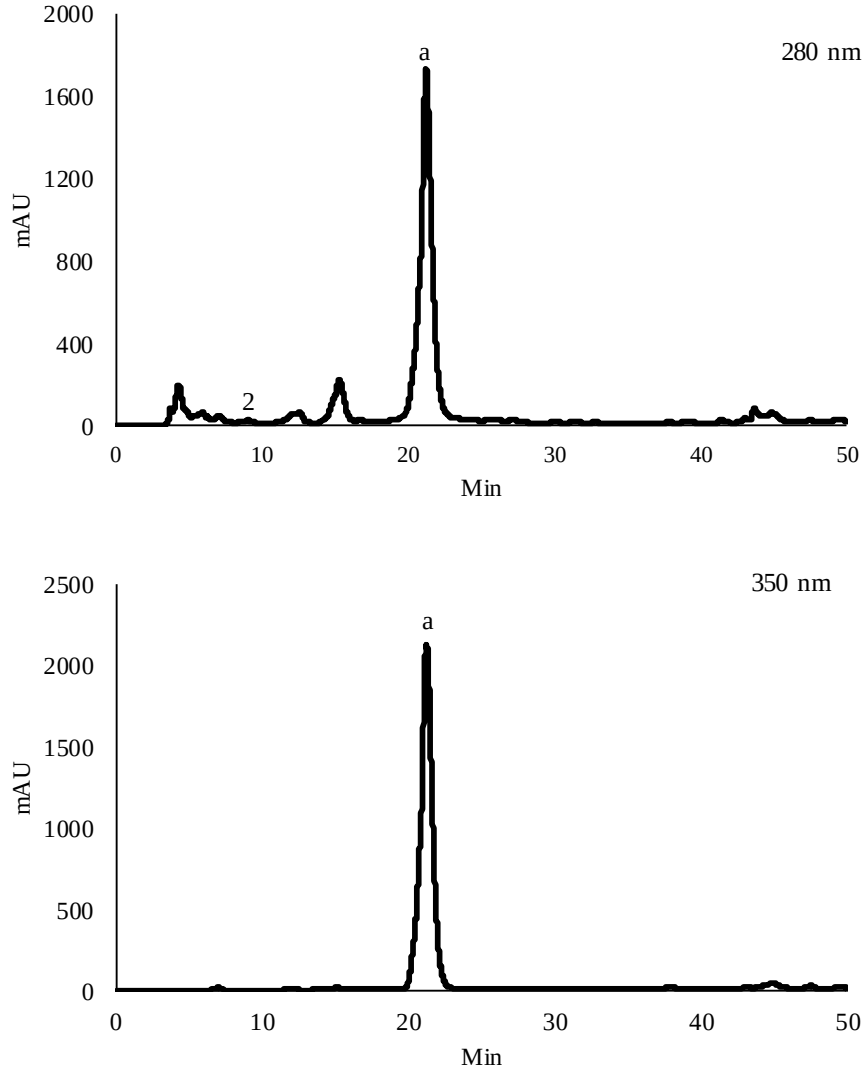
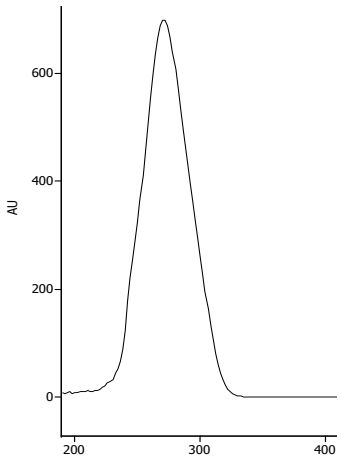
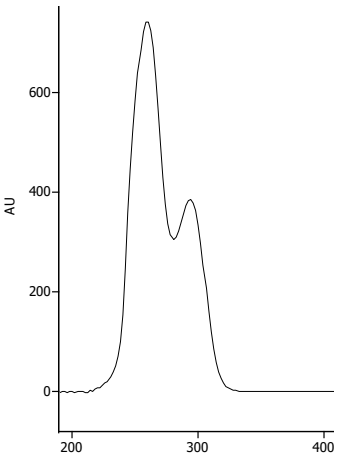


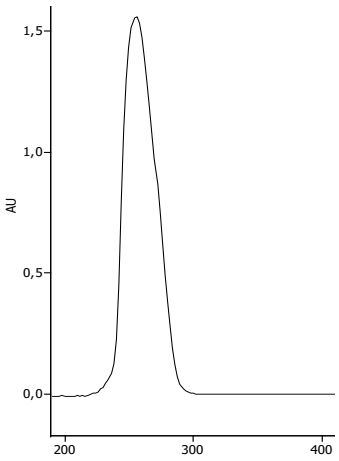
Supplementary material 2. HPLC-DAD UV-vis spectra of the identified phenolic compounds in grain legume samples.



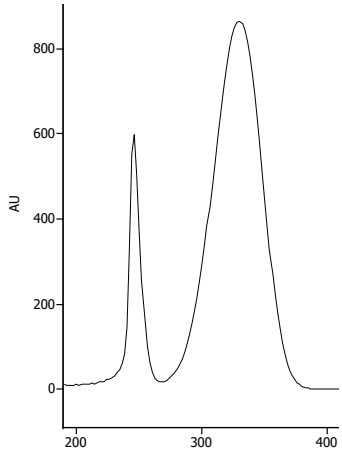
Gallic acid
(λmax = 270 nm)



Protocatechuic acid
(λmax = 259, 292 nm)



p-Hydroxybenzoic acid
(λmax = 255 nm)



Gentisic acid
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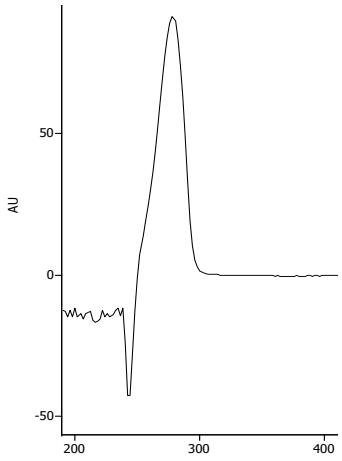
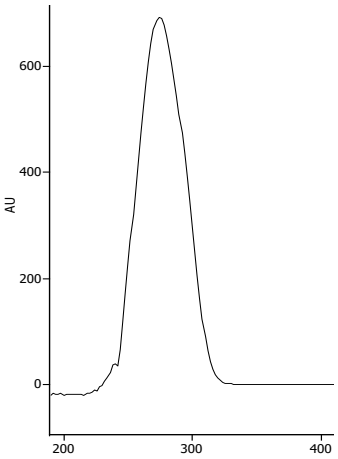
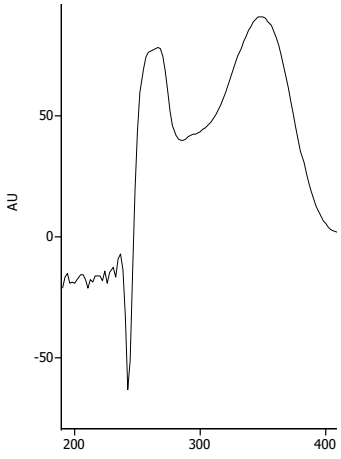


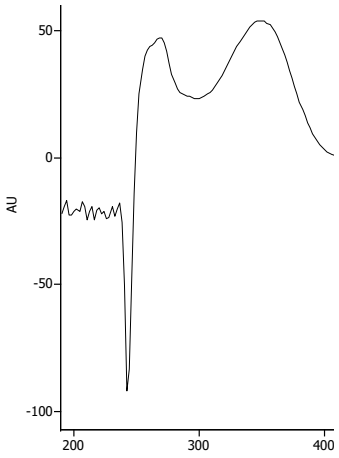
Epicatechin
(λmax = 275 nm)



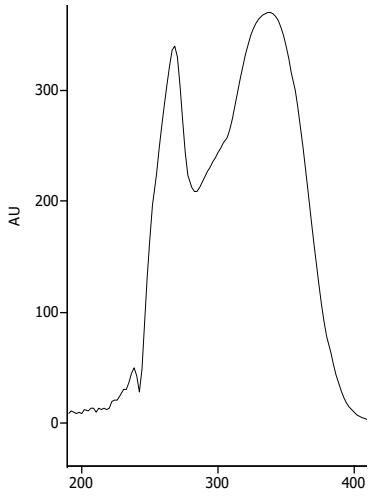
Syringic acid
(λmax = 274 nm)



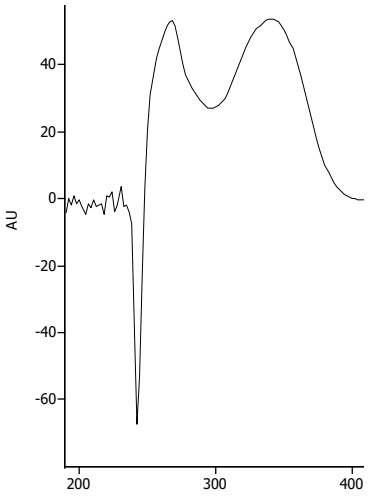
Luteolin-8-*C*-glucoside
(λmax = 257, 266, 292sh, 348 nm)



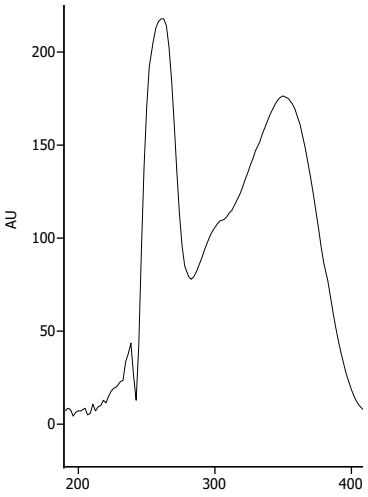
Luteolin-6-*C*-glucoside
(λmax = 257, 269, 348 nm)



Apigenin-8-*C*-glucoside
(λmax = 268, 300sh, 337 nm)



Luteolin-3,7-di-*O*-glucoside
(λmax = 268, 340 nm)



Myricetin-3-*O*-rhamnnoside
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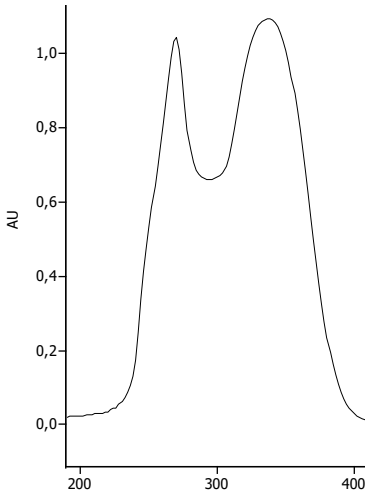
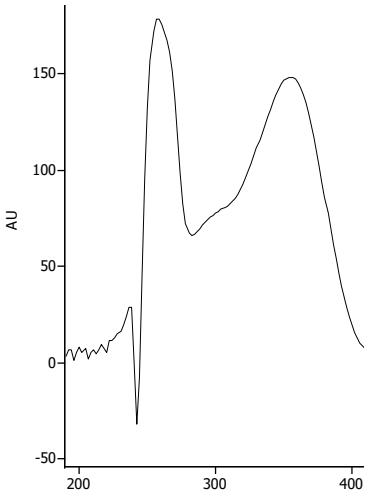
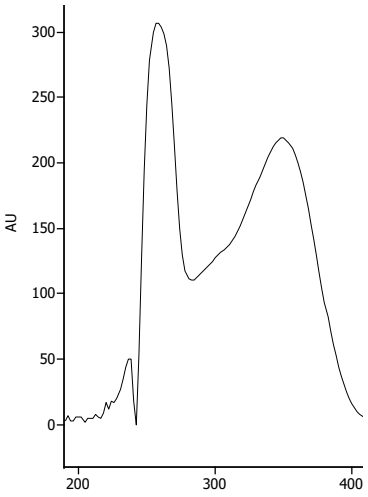


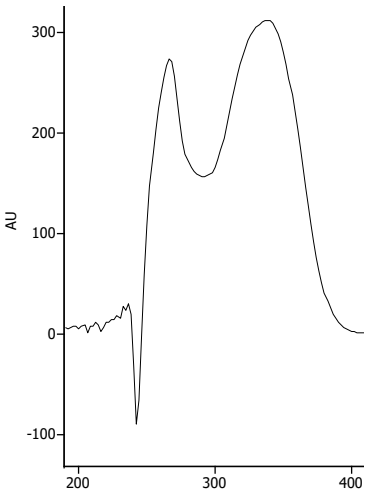
Apigenin-6-*C*-glucoside
(λmax = 270, 334 nm)



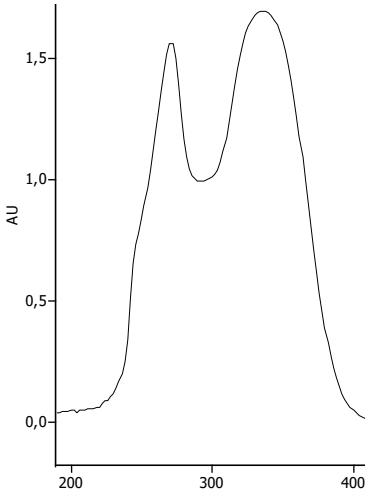
Quercetin-3-*O*-galactoside
(λmax = 257, 268sh, 298sh, 360 nm)



Quercetin-3-*O*-rhamnoside
(λmax = 256, 264sh, 301sh, 349 nm)



Apigenin-7-*O*-neohesperoside
(λmax = 266, 335 nm)



Apigenin heteroside from *Lupuinus* spp. samples
(λmax = 247sh, 271, 335 nm)

CHAPTER 5: HPLC-DAD-ESI/MS^n PROFILING OF PHENOLIC COMPOUNDS FROM *LATHYRUS CICERA* L. SEEDS

Reprinted from Food Chemistry, 214, F. Ferreres, S.C.Q. Magalhães, A. Gil-Izquierdo, P. Valentão, A.R.J. Cabrita, A.J.M. Fonseca, P.B. Andrade, HPLC-DAD-ESI/MS^n profiling of phenolic compounds from *Lathyrus cicera* L. seeds, 678-685, Copyright (2017), with permission from Elsevier.

S.C.Q. Magalhães performed seeds' phenolic compounds extraction and wrote the Introduction and Conclusion sections as well as part of the Results and discussion section.

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HPLC-DAD-ESI/MS^n profiling of phenolic compounds from *Lathyrus cicera* L. seeds

F. Ferreres [a,*], S.C.Q. Magalhães [b], A. Gil-Izquierdo [a], P. Valentão [c], A.R.J. Cabrita [b], A.J.M. Fonseca [b], P.B. Andrade [c,*]

[a] Research Group on Quality, Safety and Bioactivity of Plant Foods, Department of Food Science and Technology, CEBAS (CSIC), P.O. Box 164, 30100 Campus University Espinardo, Murcia, Spain
[b] REQUIMTE/LAQV, ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal
[c] REQUIMTE/LAQV, Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, R. Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal

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ABSTRACT

Lathyrus cicera L. seeds are of interest for food and feed purposes. Despite the recognized antioxidant activity of the seeds, arising from the phenolic fraction, their phenolic compounds have not been studied in depth yet. Therefore, to determine the phenolics profile of these seeds, a target analysis was performed using high-performance liquid chromatography coupled to photodiode-array detection and electrospray ionization/ion trap mass spectrometry (HPLC-DAD-ESI/MS^n). Thirty-seven glycosylated flavonoids were identified for the first time in the seeds of this species and, according to their MS fragmentation, clustered in flavonol-3-*O*-di-/tri-glycosides-7-*O*-rhamnosides and other flavonol-glycosides, and flavonol-3-*O*-(cinnamoyl)glycoside-7-*O*-rhamnosides, flavonol-3-*O*-(dihydrophaseoyl, cinnamoyl)glycoside-7-*O*-rhamnosides and flavonol-3-*O*-(malonyl)glycoside-7-*O*-rhamnosides. Glycosides of kaempferol were the main flavonoids found (10 non-acylated and 21 acylated), followed by those of quercetin (3) and those of isorhamnetin, apigenin and luteolin (1). The most abundant flavonols were identified as kaempferol-3-*O*-(2-hexosyl)hexoside-7-*O*-rhamnosides. The methodology used allowed to increase the knowledge on a relevant phytochemical class of seeds from *L. cicera*.

1. Introduction

Among the 160 species composing the genus *Lathyrus*, *Lathyrus cicera* L., commonly called "chickling vetch", is a species with major economic importance for animal consumption (poultry, pig and sheep; Patto & Rubiales, 2014; White et al., 2002), being cultivated since ancient times and domesticated in Southern France and in the Iberian Peninsula (Patto & Rubiales, 2014). Indeed, *L. cicera* seeds are cheap sources of protein and other nutrients (Pastor-Cavada, Pastor, Juan, & Vioque, 2013). In addition, the high agronomic resilience of *Lathyrus* species, in general, and the already existing low (0.09–0.49%) neurotoxin 3-(-*N*-oxalyl)-L-2,3-diamino propionic acid (ODAP) lines of *L. cicera* are two factors considered of interest in a scenario where global demand for food is predicted to double by 2050 in order to meet population growth (Patto & Rubiales, 2014).

In terms of proximate composition, these seeds present similarities to other commonly used grain legumes (e.g. field peas and faba beans), containing about 25% protein (Hanbury, White, Mullan, & Siddique, 2000; Pastor-Cavada, Juan, Pastor, Alaiz, & Vioque, 2014). Besides the interesting nutritive value, *L. cicera* seeds, as all *Lathyrus* species, are potential sources of functional compounds, such as antioxidant phenolics (Pastor-Cavada, Juan, Pastor, Alaiz, & Vioque, 2009). Those authors studied the antioxidant activity of 15 wild *Lathyrus* species and found that they contained phenolic compounds with a high antioxidant activity when compared with commercial legumes like chickpeas (*Cicer arietinum* L.), narrow-leafed lupins (*Lupinus angustifolius* L.) or soybeans (*Glycine max* L.). They also reported *L. cicera* among the *Lathyrus* species with the highest antioxidant activity. Yet, studies on the polyphenols' profile of seeds from *Lathyrus* spp. are scarce and restricted to a few species, such as *L. sativus* L. or *L. maritimus* L. (Chavan, Amarowicz, & Shahidi, 1999; Chavan, McKenzie, Amarowicz, & Shahidi, 2003; Fratianni et al., 2014; Pastor-Cavada et al., 2009; Ranabahu & Harborne, 1993; Wang et al., 1998). More than two decades ago, Ranabahu and Harborne (1993) studied the

* Corresponding authors.
E-mail addresses: federico@cebas.csic.es (F. Ferreres), pandrade@ff.up.pt (P.B. Andrade).

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0308-8146/

flavonoids profile of leaves, flowers, seeds and pods of thirty-eight *Lathyrus* species, having only found, by thin-layer chromatography (TLC), quercetin in the seeds of *L. cicera*. Although used since the early 1960s in flavonoid analysis, TLC is especially suitable for rapid screening tests prior to detailed analysis by instrumental chromatographic techniques (de Rijke et al., 2006; de Villiers, Venter, & Pasch, 2016). As far as we are aware of, there is no other report on the phenolic compounds of seeds of this species.

In this context, the aim of the present work was to determine, for the first time, the phenolics profile of mature raw seeds from *L. cicera* by high performance liquid chromatography-photodiode-array detection-electrospray ionization ion trap/tandem mass spectrometry (HPLC-DAD-ESI/MS^n). This methodology benefits the analysis of polyphenols in samples of *L. cicera* seeds with selectivity, sensitivity and rapidity (Abad-Garcia, Garmon-Lobato, Berrueta, Gallo, & Vicente, 2012; Truchado, Vit, Ferreres, & Tomas-Barberan, 2011). A deeper knowledge on the phenolic compounds composition of *L. cicera* seeds could increase the interest on them also for food purposes and, therefore, favour the extension of the cultivation of this crop.

2. Material and methods

2.1. Plant material

Mature raw seeds of *Lathyrus cicera* L. var. Grão-da-gramicha (a lot sown in Elvas, Portugal, in the campaign year of 2012/2013), provided by Instituto Nacional de Investigação Agrária e Veterinária, I.P. (Oeiras, Portugal), were dried in a forced-air oven (65 °C, 24 h) and grounded in a mill equipped with a sieve of 1-mm for analysis.

2.2. Reagents and solid-phase extraction columns

Methanol (MeOH) of LiChrosolv grade was purchased from Merck (Darmstadt, Germany), hydrochloric acid 37% (HCl) from VWR Chemicals (Radnor, PA) and potassium hydroxide (KOH; ⩾85%) from Sigma-Aldrich (St. Louis, MO). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA). The C18 non-endcapped columns (50 µm particle size, 60 Å porosity; 10 g of sorbent mass/70 mL of reservoir volume) were obtained from Chromabond (Macherey-Nagel, Germany).

2.3. Phenolics extraction

Extraction followed the procedure previously described (Taveira et al., 2009) with slight modifications. Five grams of sample were sonicated for 15 min and stirred (300 rpm) for 30 min, at 30 °C, with 50 mL of 50% MeOH. Despite 80% acetone showed to be more efficient in extracting polyphenols from seeds of *L. maritimus* than 80% methanol and 80% ethanol (Chavan & Amarowicz, 2013), the suitable polarity of MeOH (together with that of water) is recognized to allow the recovery of a wide range of polyphenols with diverse structures and the reduction of polyphenoloxidase activity (Abad-García et al., 2007; Ferreres et al., 2011). The extract was centrifuged (10 min, 4000 rpm) and the material was re-extracted with 50 mL of 50% methanol under the same conditions. The combined supernatants were evaporated to dryness under reduced pressure, at 30 °C, in a Buchi Rotavapor® R-215, equipped with a vacuum controller V-850 and a vacuum pump V-700 (Flawil, Switzerland). The residue was dissolved in 50 mL of water acidified to pH 2 with HCl. The solution obtained was applied in the C18 column, previously conditioned with 70 mL of MeOH and 30 mL of acidified water. Polar compounds were discarded with the aqueous solvent and the retained phenolic compounds were then eluted with 50 mL of MeOH. The eluate was concentrated by rotary evaporation, under reduced pressure at 30 °C, using the equipment referred above. The residue obtained was redissolved in appropriate volume of MeOH of LiChrosolv grade (ca. 2 mL) and membrane-filtered (0.45 µm) for further analysis by HPLC-DAD-ESI/MS^n (native extract).

2.4. Alkaline hydrolysis

Alkaline hydrolysis was carried out by adding 3 M KOH to the hydromethanolic extract concentrated in phenolics until pH ~9–10. The mixture was kept at room temperature in a stoppered test tube for 2 h. Then, the hydrolysis products were acidified with concentrated HCl to pH ~1–2 and directly analysed by HPLC-DAD-ESI/MS^n.

2.5. HPLC-DAD-ESI/MSn qualitative analysis of phytochemical compounds

Chromatographic analysis was carried out on a 150 mm × 4.6 mm, 5 µm, Kinetex 100 Å RP-18 column, with a 2 mm × 4.6 mm i.d., 2 µm guard column of the same material (Phenomenex, Macclesfield, UK). The mobile phase consisted of two solvents: water-formic acid (1%) (A) and acetonitrile (B), starting with 10% B and using a gradient to obtain 30% B at 20 min, 50% at 25 min, 60% at 27 min. The flow rate was 800 µL min^{-1} and the injection volume 8 µL. Spectral data from all peaks were accumulated in the range of 240–400 nm and chromatograms were recorded at 280 and 330 nm. The analysis was carried out in an Agilent HPLC 1200 series (Agilent Technologies, Waldbronn, Germany). The ionization conditions were adjusted at 350 °C and 4.0 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L min^{-1}, respectively. The full scan mass covered the range from *m/z* 100 up to *m/z* 1500. Mass spectrometry data were acquired in the negative ionization mode.

3. Results and discussion

The screening of the hydromethanolic extract concentrated in phenolics of mature raw seeds of *L. cicera* by HPLC-DAD-ESI/MS^n revealed an UV chromatogram (330 nm; Fig. 1) presenting two major peaks (**4** and **5**) with UV spectra of kaempferol substituted at position 3, and several other minor peaks, most of them also flavonols (mainly kaempferol or quercetin), equally glycosylated at position 3 (Mabry, Markham, & Thomas, 1970). Other peaks suggested the presence of acylation with cinnamic acids, since their UV spectra were formed by overlapping the spectra of both the acid and the flavonoid, resulting in a band I (~315–330 nm) with high absorption with respect to band II (268 nm) and hypsochromically displaced relatively to the UV spectrum of a non-acylated flavonoid. Other acylated flavonoids presenting an atypical absorption of band II (268 nm) with respect to the band I (348 nm), along with other possible acylated derivatives, could also be observed. For this reason, an alkaline hydrolysis was carried out to yield an extract in which the acylated derivatives disappear (Fig. 1), being therefore possible to confirm their presence.

Compounds were grouped according to their MS fragmentation in flavonol-3-*O*-di-/tri-glycosides-7-*O*-rhamnosides and other flavonol-glycosides (Table 1), and flavonol-3-*O*-(cinnamoyl) glycoside-7-*O*-rhamnosides, flavonol-3-*O*-(dihydrophaseoyl, cinnamoyl)glycoside-7-*O*-rhamnosides and flavonol-3-*O*-(malonyl) glycoside-7-*O*-rhamnosides (Table 2). Indeed, flavonoids are dominant phenolic compounds in legume seeds, occurring mostly as glycosides (Amarowicz & Pegg, 2008). No phenolic acid was herein identified in the hydromethanolic extract concentrated in phenolics of *L. cicera* seeds, agreeing with findings of Chavan

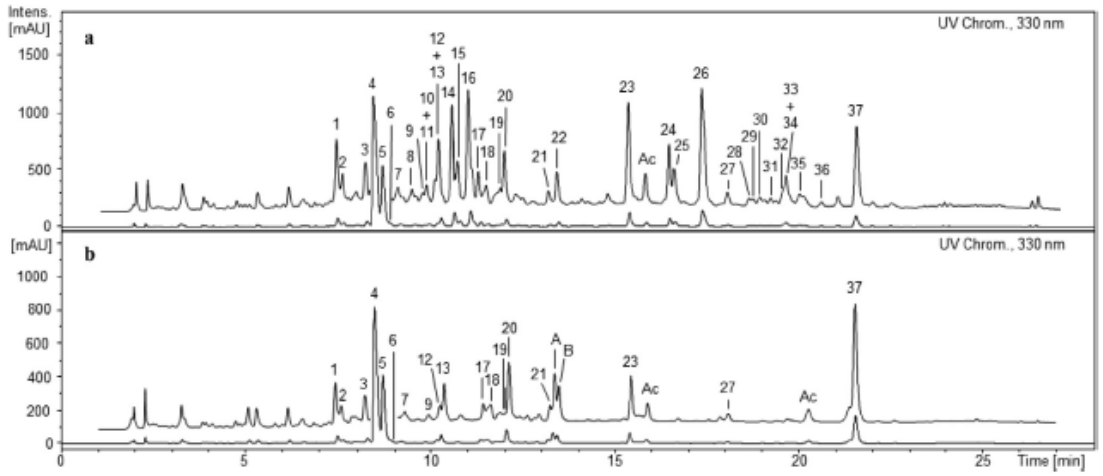


Fig. 1. HPLC-UV (330 nm) profile of native (a) and saponified extract (b) of seeds of *L. cicera*. Identity of compounds as in Tables 1 and 2. Ac: uncharacterized cinnamoyl acid derivative.

et al. (1999) with beach peas (*L. maritimus*). Those authors found phenolic acids derivatives to be absent or at low amounts, suggesting flavonoids as the main phenolics in these seeds. In contrast, Fratianni et al. (2014) could detect phenolic acids like gallic, chlorogenic, caffeic, coumaric and ferulic acids in the seeds of *L. sativus*, besides catechin, epicatechin, quercetin-3-*O*-rutinoside and quercetin-3-*O*-galactoside.

3.1. Flavonol-3-O-di-/tri-glycosides-7-O-rhamnosides

This group of compounds (**1–7**, **9**, **12**, **13** and **19**), with UV spectra of flavonols with 3-position blocked, showed a MS^2 fragmentation with loss of a fragment of 146 amu (rhamnosyl radical), yielding the base peak as practically the unique ion (Table 1, Scheme 1). Other ions could also be observed (deprotonated ions of the aglycone or resulting from the break of the interglycosidic linkage), with low relative abundance (not shown in Table 1 or Scheme 1). This type of sugar fragmentation, in which the loss of that fragment with water (146+18) is not detected, indicates a link to a phenolic hydroxyl (Cuyckens, Ma, Pocsfalvi, & Claeysi, 2000). The deprotonated ion of the aglycone was detected in high abundance in the MS^3 fragmentation of the flavonoid that had already lost the rhamnosyl radical ($MS^3[(M-H)\rightarrow(M-H-146)]^-$), being the base peak most of the times. It was also possible to observe losses of

Table 1
Rt, UV and MS$[M-H]^-$, $MS^2[M-H]^-$ and $MS^3[(M-H)\rightarrow(M-H-146)]^-$ data of non-acylated flavonoids from seeds of *Lathyrus cicera*.[a]

	Compounds[b]	*Rt* (min)	Flavonol-3-*O*-di-/tri-glycoside-7-*O*-rhamnosides								
			UV (nm)	$[M-H]^-$, *m/z*	$MS^2[M-H]^-$, *m/z* (%) −146	$MS^3[(M-H)\rightarrow(M-H-146)]^-$, *m/z* (%) −132	−150	−162	−180	−266	[Aglc−H/2H]$^-$
1	Qct-3-(2Hx)Hx-7-Rh	7.5	254, 266sh, 354	771	625			463(18)	445(23)		301(100)
2	Qct-3-(2Glc)Glc-7-Rh	7.7	254, 266sh, 352	771	625			463(16)	445(8)		300(100)
3	Kpf-3-(2Glc, 6Rh)Glc-7-Rh	8.2	266, 344	901	755			593(10)	575(100)	489(7)	285(95)
4	Kpf-3-(2Hx)Hx-7-Rh	8.4	266, 318sh, 347	755	609			447(3)	429(35)		285(100)
5	Kpf-3-(2Glc)Glc-7-Rh	8.6	266, 320sh, 348	755	609			447(4)	429(60)		285(100)
6	Qct-3-(2Xyl)Gal-7-Rh	8.8	—[c]	741	595	463(25)	445(60)				300(100)
7	Isrh-3-(2Glc)Glc-7-Rh	9.1	255, 266sh, 352	785	639			477(5)	549(20)		315(100)
9	Kpf-3-(2Xyl, 6Rh)Gal-7-Rh	9.8	266, 342	871	725	593(15)	575(100)			459(20)	285(70)
12	Kpf-3-(2Xyl)Gal-7-Rh	10.2	—[c]	725	579	447(4)	429(30)				285(100)
13	Kpf-3-(2Xyl)Glc-7-Rh	10.3	266, 316sh, 348	725	579	447(20)	429(60)				285(100)
19	Kpf-3(6Rh)Gal-7-Rh	12.0	—[c]	739	593					327(5)	285(100)
			Other flavonoid-glycosides								
							$MS^2[M-H]^-$, *m/z* (%) −146	−162	−164	−180	[Aglc−H/2H]$^-$
17	Kpf-3Gal-7-Rh	11.3	266, 316sh, 348	593			447(100)	431(5)			285(10)
18	Kpf-3-(2Glc)Glc	11.7	—[c]	609				447(16)		429(15)	285(100)
20	Kpf-3-Glc-7Rh	12.1	266, 318sh, 348	593			447(100)	431(35)			285(40)
21	Lut-7-(2Rh)Glc	13.2	254, 266sh, 346	593			447(30)		429(3)		285(100)
23	Apg-7-(2Rh)Glc	15.4	266, 338	577					413(4)		269(100)
27	Qct-7-Rh	18.0	255, 266sh, 368	447							301(100)

[a] Main observed fragments. Other ions were found but they have not been included.
[b] Qct: quercetin; Kpf: kaempferol; Isrh: isorhamnetin; Lut: luteolin; Apg: apigenin; Hx: hexose; Pt: pentose; Rh: rhamnose; Glc: glucose; Gal: galactose; Xyl: xylose.
[c] Compound was found at trace amounts or co-eluted with others, which did not allow observing its UV spectrum.

Table 2
Rt, UV and MS[M−H]⁻, MS^2[M−H]⁻ and MS^3[(M−H)→(M−H−146)]⁻ data of acylated flavonoids from seeds of *Lathyrus cicera*.[a]

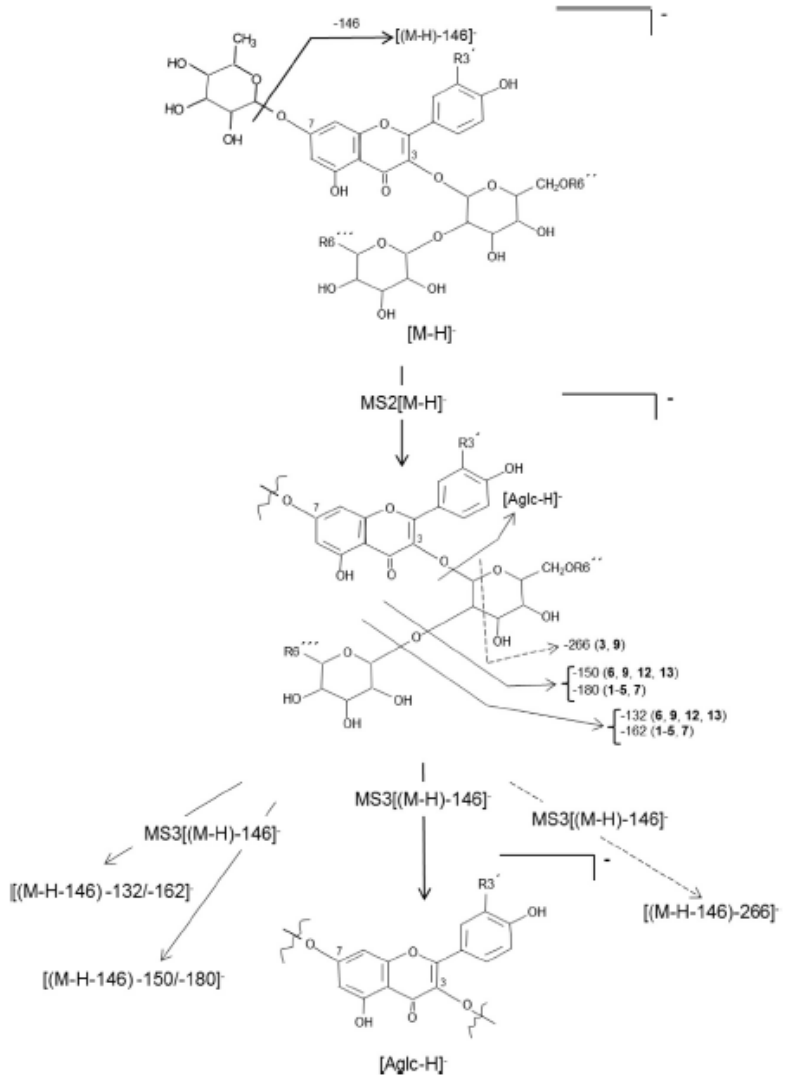
	Compounds[b]	Rt (min)	UV (nm)	[M−H]⁻, m/z	MS^2[M−H]⁻, m/z (%)			MS^3[(M−H)→(M−H−146)]⁻, m/z (%)			
					−146	−146−Acyl	[Aglc−H/2H]⁻	−Acyl (Ac)	−Ac−162	−Ac−180	[Aglc−H/2H]⁻
	Flavonol-3-*O*-(cinnamoyl)glycoside-7-*O*-rhamnosides										
10	Kpf-3-[6Snp(2Hx)]Hx-7-Rh	9.9	—[c]	961	815(100)	609(6)		609(100)[d]		429(10)	285(12)
14	Kpf-3-[6Frl(2Hx)]Hx-7-Rh	10.6	268, 296sh, 332	931	785(100)	609(7)		609(100)[d]	447(2)	429(5)	285(15)
15	Kpf-3-[6Frl(2Hx)]Hx-7-Rh isomer	10.8	268, 290sh, 332	931	785(100)	609(3)	285(1)	609(100)[d]		429(5)	285(7)
16	Kpf-3-[6p-Co(2Hx)]Hx-7-Rh	11.0	268, 292sh, 316	901	755(100)	609(2)		609(100)[d]		429(4)	285(10)
A	Kpf-3-[6Frl(2Hx])]Hx-7-Rh isomer	13.3	268, 298sh, 326	931	785(100)	609(4)		609(100)[d]	447(2)	429(8)	285(16)
B	Kpf-3-[6p-Co(2Hx)]Hx-7-Rh isomer	13.4	268, 314, 356sh	901	755(100)	609(5)		609(100)[d]	447(1)	429(5)	285(10)
31	Kpf-3-[2p-Co(2Hx)]Hx-7-Rh	19.2	—[c]	901	755(42)	609(100)	285(17)	609(100)[d]	447(45)		284(40)
32	Kpf-3-[2p-Co(2Hx)]Hx-7-Rh isomer	19.5	—[c]	901	755(80)	609(100)	285(20)	609(100)[d]		429(20)	284(15)
35	Kpf-3-[2Frl(2Hx)]Hx-7-Rh	20.0	—[c]	931	785(85)	609(100)	285(5)	609(100)[d]		429(35)	285(20)
	Flavonol-3-*O*-(DPA, cinnamoyl)glycoside-7-*O*-rhamnosides										
					MS^2[M−H]⁻, m/z (%)						
						−146	−264	−410[e]	−586/−556[e]		
24	Kpf-3-[6DP(2Hx)]Hx-7-Rh	16.5	268, 348	1019		873(60)	755(4)	609(100)			
25	Kpf-3-[6DP(2Hx)]Hx-7-Rh isomer	16.6	268, 348	1019		873(100)	755(5)	609(95)			
26	Kpf-3-[6DP(2Hx)Hx]-7-Rh isomer	17.4	268, 348	1019		873(100)	755(6)	609(75)			
28	Kpf-3-[6DP(2Hx)](6Frl)Hx-7-Rh	18.6	—[c]	1195		1049(20)	931(10)	785(100)	609(10)		
29	Kpf-3-[2DP(2Xyl)]Hx-7-Rh	18.7	—[c]	989		843(70)	579(100)				
30	Kpf-3-[6DP(2Hx)](6p-Co)Hx-7-Rh	18.9	—[c]	1165		1019(5)	901(10)	755(100)	609(16)		
33	Kpf-3-[6DP(2Hx)](6Frl)Hx-7-Rh isomer	19.6	—[c]	1195		1049(30)	931(10)	785(100)	609(18)		
34	Kpf-3-[6DP(2Hx)](6p-Co)Hx-7-Rh	19.7	—[c]	1165		1019(7)	901(5)	755(100)	609(10)		
36	Kpf-3-(6DP)Hx-7-Rh	20.6	268, 348	857		711(2)	593(2)	447(100)			
	Flavonol-3-*O*-(malonyl)glycoside-7-*O*-rhamnosides										
					MS^2[M−H]⁻, m/z (%)			MS^3[(M−H)→(M−H−44−146]⁻, m/z (%)			
					−44	−44−146		−42	−42−180	[Aglc−H/2H]⁻	
8	Kpf-3-[2Mln(2Hx)]Hx-7-Rh	9.5	265, 320sh, 350	841	797(100)	651(30)[e]		509(90)	429(55)	285(100)	
11	Kpf-3-[6Mln(6Hx)]Hx-7-Rh	9.9	—[c]	841	797(100)	651(30)[e]				285(100)	
22	Kpf-3-(6Mln)Hx-7-Rh	13.5	266, 318sh, 348	679	635(100)	489(10)				285(100)	

[a] Main observed fragments. Other ions were found but they have not been included.
[b] Kpf: kaempferol; Hx: hexosyl; Xyl: xylosyl; Rh: rhamnosyl; Snp: sinapoyl; Frl: feruloyl; *p*-Co: *p*-coumaroyl; DP: dihydrophaseoyl; Mln: malonyl.
[c] Compound was found at trace amounts or co-eluted with others, which did not allow observing its UV spectrum.
[d] Other ions from MS^3. **10**: 623(85), 591(15); **14**: 623(80), 591(30); **15**: 623(50), 591(15); **16**: 591(20); **A**: 623(45), 591(5); **B**: 591(5); 35: 623(95), 591(30).
[e] −410: −(146+264); −586/556: −(146+264+Ac(176/146)).

−162/−180 amu (**1**, **2**, **4**, **5** and **7**) or −132/−150 amu (**6**, **12** and **13**) that indicate an interglycosidique linkage (hexosyl or pentosyl, respectively) different from 1→6, probably 1→2 (Cuyckens et al., 2000; Scheme 1). Therefore, these compounds are formed by a rhamnose and a disaccharide, hexosyl(1→2)hexoside (**1**, **2**, **4**, **5** and **7**) or pentosyl(1→2)hexoside (**6**, **12** and **13**), linked to two different phenolic hydroxyl groups: one at position 3, the other most common glycosylation position being the 7 (Fig. 2). This is confirmed by the study of Ranabahu and Harborne (1993) in thirty-eight species of *Lathyrus* and by that of Ohtsuki, Murai, Iwashina, and Setoguchi (2013) in the leaves of *L. japonicus* L. In flavonol-3,7-diglycosides the preferential fragmentation position is at 7 (Fratianni et al., 2014). Therefore, these compounds can be labelled as 3-*O*-(2-hexosyl)hexoside-7-*O*-rhamnoside linked to quercetin (**1** and **2**), kaempferol (**4** and **5**) and isorhamnetin (**7**), and 3-*O*-(2-pentosyl)hexoside-7-*O*-rhamnoside linked to quercetin (**6**) and kaempferol (**12** and **13**).

Compounds **3** and **9** exhibited a deprotonated molecular ion 146 amu higher than that of **4**/**5** and **12**/**13**, respectively (901 and 871 *vs* 755 and 725), and presented a MS^3 fragmentation similar to that of **4**/**5** and **12**/**13**, respectively, with an additional loss of a 266 amu fragment (120+146), resulting from the intern rupture of the hexose linked to a rhamnose (146 amu) at its 6-position, by positions 0, 2 (fragment of 120 amu) (Scheme 1), since the linkage 1→6 is very stable. Therefore, these compounds would be kaempferol-3-*O*-(2-hexosyl, 6-rhamnosyl)hexoside-7-*O*-rhamnoside (**3**) and kaempferol-3-*O*-(2-pentosyl, 6-rhamnosyl) hexoside-7-*O*-rhamnoside (**9**).

For compound **19**, the MS^3 fragmentation of the rhamnosylhexoside linked to the hydroxyl at 3 does not yield ions resulting from the rupture of the interglycosidic linkage. Instead, it is observed the loss of the fragment of 266 amu, which, as referred above, indicates a rhamnosylation at position 6 of an hexose. This compound would be kaempferol-3-*O*-(6-rhamnosyl)hexoside-7-*O*-rhamnoside.



Scheme 1. MS fragmentation pathway of flavonol-3-*O*-(2-glycosyl)hexoside-7-*O*-rhamnoside derivatives from seeds of *L. cicera*.

3.2. Other flavonoid-glycosides

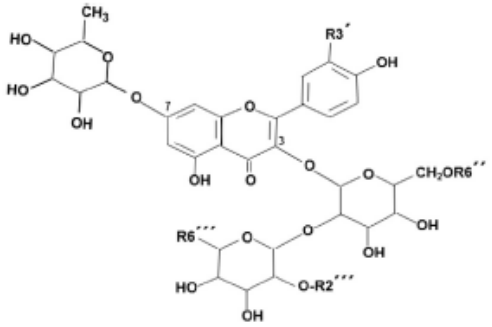
This group includes other flavonoids with a lower degree of glycosylation. Compounds **17** and **20** present a mass 162 amu lower than that of **4**/**5** (593 *vs* 755). Their MS^2 fragmentation showed losses of −146 and −162 amu, without the corresponding +18 ions. Therefore, they present a rhamnose and a hexose linked to two phenolic hydroxyl groups and could be considered derivatives of **4**/**5** that had lost the terminal hexose. Hence, they could be labelled as kaempferol-3-*O*-hexoside-7-*O*-rhamnosides.

Compound **18**, with 146 amu inferior to **4**/**5**, had a MS^2 fragmentation similar to the MS^3 fragmentation of **4**/**5**. Therefore, similarly to what was exposed above, we could consider it a derivative of **4**/**5**, in which there was no substitution at 7, being labelled as kaempferol-3-*O*-(2-hexosyl)hexoside.

Compound **27**, having UV spectrum of quercetin with free hydroxyl at position 3 (band I 368 nm) and mass corresponding to a quercetin-rhamnoside, is a derivative of **1**/**2** without the glycosidic fraction at 3-position, namely quercetin-7-*O*-rhamnoside.

Two other compounds (**21** and **23**) are rhamnosyl(1→2)hexosides (losses of −146 and/or −164) derivatives of the flavones luteolin and apigenin (UV spectra and $[Aglc-H]^-$), being labelled as luteolin-7-*O*-(2-rhamnosyl)hexoside (**21**) and apigenin-7-*O*-(2-rhamnosyl)hexoside (**23**) (Table 1).

While studying several vegetal tissues of species of *Lathyrus*, Ranabahu and Harborne (1993) found free quercetin and kaempferol in the leaves and free quercetin in the seeds of *L. cicera*. Regarding glycosides, those authors have found kaempferol-3-*O*-sophoroside-7-*O*-glucoside and kaempferol-3-*O*-robinoside-7-*O*-rhamnoside in the leaves. In relation to the compounds detected herein, they also detected kaempferol-3-*O*-(2-xylosyl)galactoside-7-*O*-rhamnoside and kaempferol-3-*O*-glucoside-7-*O*-rhamnoside, among others, in other species. On the other hand, they indicated that the flavonol patterns could be split into four types, according to the nature of the disaccharide linked to the hydroxyl at position 3: sophorose(glucosyl 1→2 glucose), lathyrose(xylosyl 1→2 galactose), robinobiose(rhamnosyl 1→6 galactose) and rutinose(rhamnosyl 1→6 glucose). Besides the aglycones above mentioned,



Compounds		R3′	R6″	R2‴	R6‴
Flavonol-3-*O*-(2-glycosyl)hexoside-7-*O*-rhamnosides					
1	Qct-3-(2Hx)Hx-7-Rh	OH	H	H	CH_2OH
2	Qct-3-(2Glc)Glc-7-Rh	OH	H	H	CH_2OH
3	Kpf-3-(2Glc, 6Rh)Glc-7-Rh	H	Rh	H	CH_2OH
4	Kpf-3-(2Hx)Hx-7-Rh	H	H	H	CH_2OH
5	Kpf-3-(2Glc)Glc-7-Rh	H	H	H	CH_2OH
6	Qct-3-(2Xyl)Gal-7-Rh	H	H	H	H
7	Isrh-3-(2Glc)Glc-7-Rh	OCH_3	H	H	CH_2OH
9	Kpf-3-(2Xyl, 6Rh)Gal-7-Rh	H	Rh	H	H
12	Kpf-3-(2Xyl)Gal-7-Rh	H	H	H	H
13	Kpf-3-(2Xyl)Glc-7-Rh	H	H	H	H
Kaempferol-3-*O*-[cinnamoyl(2-hexosyl)]hexoside-7-*O*-rhamnosides					
10	Kpf-3-[6Snp(2Hx)]Hx-7-Rh	H	H	H	CH_2O-Snp
14	Kpf-3-[6Frl(2Hx)]Hx-7-Rh	H	H	H	CH_2O-Frl
15	Kpf-3-[6Frl(2Hx)]Hx-7-Rh isomer	H	H	H	CH_2O-Frl
16	Kpf-3-[6*p*-Co(2Hx)]Hx-7-Rh	H	H	H	CH_2O-*p*-Co
A	Kpf-3-[6Frl(2Hx])]Hx-7-Rh isomer	H	H	H	CH_2O-Frl
B	Kpf-3-[6*p*-Co(2Hx)]Hx-7-Rh isomer	H	H	H	CH_2O-*p*-Co
31	Kpf-3-[2*p*-Co(2Hx)]Hx-7-Rh	H	H	*p*-Co	CH_2OH
32	Kpf-3-[2*p*-Co(2Hx)]Hx-7-Rh isomer	H	H	*p*-Co	CH_2OH
35	Kpf-3-[2Frl(2Hx)]Hx-7-Rh	H	H	Frl	CH_2OH
Kaempferol-3-*O*-(DPA,cinnamoyl)[2-glycosyl)hexoside]-7-*O*-rhamnosides					
24	Kpf-3-[6DP(2Hx)]Hx-7-Rh	H	H	H	CH_2O-DP
25	Kpf-3-[6DP(2Hx)]Hx-7-Rh isomer	H	H	H	CH_2O-DP
26	Kpf-3-[6DP(2Hx)Hx]-7-Rh isomer	H	H	H	CH_2O-DP
28	Kpf-3-[6DP(2Hx)](6Frl)Hx-7-Rh	H	Frl	H	CH_2O-DP
29	Kpf-3-[2DP(2Xyl)]Hx-7-Rh	H	H	DP	H
30	Kpf-3-[6DP(2Hx)](6*p*-Co)Hx-7-Rh	H	*p*-Co	H	CH_2O-DP
33	Kpf-3-[6DP(2Hx)](6Frl)Hx-7-Rh isomer	H	Frl	H	CH_2O-DP
34	Kpf-3-[6DP(2Hx)](6*p*-Co)Hx-7-Rh	H	*p*-Co	H	CH_2O-DP

Fig. 2. Chemical structures of different flavonoids found in the seeds of *L. cicera*.

apigenin and luteolin were also found by them in other species. Few more studies have been performed on the flavonoids characterization of *Lathyrus*, but we are not aware of none on *L. cicera*.

Therefore, for a deeper characterization of the compounds noticed in the present work, and given the reversed-phase chromatographic mobility, we can tentatively indicate that in the pairs of isomers **1**/**2** and **4**/**5**, differing only in their aglycones, the hexosyl(1→2)hexoside linked to the 3-hydroxyl of compounds **2** and **5** may be sophoroside. The compounds would be quercetin-3-*O*-sophoroside-7-*O*-rhamnoside (**2**) and kaempferol-3-*O*-sophoroside-7-*O*-rhamnoside (**5**), whereas, in **1** and **4** a galactose would be involved. Compound **7** could be isorhamnetin-3-*O*-sophoroside-7-*O*-rhamnoside. Likewise, regarding compounds **12**/**13**, the one eluting first may correspond to kaempferol-3-*O*-lathyroside-7-*O*-rhamnoside (kaempferol-3-*O*-(2-xylosyl)galacto side-7-O-rhamnoside) (**12**), detected in several *Lathyrus* species, and compound **13** to kaempferol-3-*O*-(2-xylosyl)glucoside-7-*O*-rhamnoside. Compound **6** differs from **12** in the aglycone and could be labelled as quercetin-3-*O*-lathyroside-7-*O*-rhamnoside (**6**). In compound **19**, the rhamnosyl(1→6)hexoside may correspond to the robinoside detected in other species and its structure would be kaempferol-3-*O*-robinoside-7-*O*-rhamnoside. The triglycoside of compound **3** could derive from **5** by the addition of a rhamnose at position 6; thus, compound **3** would be kaempferol-3-*O*-(6-rhamnosyl)sophoroside-7-*O*-rhamnoside (**3**). Similarly, compound **9** could derive from **12** by rhamnosylation at position 6, corresponding, therefore, to kaempferol-3-*O*-(2-xylosyl-6-rhmanosyl)-

galactoside-7-*O*-rhamnoside (**9**) (Fig. 2). The other glycosides tentatively identified would be kaempferol-3-*O*-galactoside-7-*O*-rhamnoside (**17**), kaempferol-3-*O*-glucoside-7-*O*-rhamnoside (**20**), kaempferol-3-*O*-sophoroside (**18**), luteolin-7-*O*-(2-rhamnosyl) glucoside (**21**), apigenin-7-*O*-(2-rhamnosyl)glucoside (**23**) and quercetin-7-*O*-rhamnoside (**27**).

The remaining compounds were not found in the UV chromatogram of the saponified extract, probably being acylated derivatives. As discussed below, with the exception of three compounds (**22**, **29** and **36**), they are all kaempferol-3-*O*-(2-hexosyl) hexoside-7-*O*-rhamnoside acyl derivatives, i.e. derivatives of the two major compounds (**4**/**5**). When proposing their structures, we do not take into account the nature of their hexoses, for which they yield several isomers, other than those of position of substitution.

3.3. Flavonol-3-O-(cinnamoyl)glycoside-7-O-rhamnosides

As indicated above, this group of compounds (**10**, **14**–**16**, **31**, **32** and **35**) is characterized by the presence of an UV spectrum typical of flavonoids acylated with cinnamic acids, in which band I suffers an hypsochromic displacement (~315–330 nm) and exhibits an high absorption with respect to band II. These compounds can be considered as derivatives of **4**/**5** (kaempferol-3-*O*-(2-hexosyl) hexoside-7-*O*-rhamnoside, $[M-H]^-$, *m*/*z* 755) by addition of a cinnamoyl radical: synapoyl (*m*/*z* 206: **10**), feruloyl (*m*/*z* 176: **14**, **15** and **35**) and *p*-coumaroyl (*m*/*z* 146: **16**, **31** and **32**). In their MS^2 fragmentations it was observed the loss of a fragment of 146 amu (rhamnosyl radical), to yield an ion with high relative abundance that is the base peak in most of the cases (**10**, **14**–**16**). The ions resulting from the loss of both rhamnosyl radical (−146) and the corresponding acyl were also observed. In the case of compounds **10** and **14**–**16** these ions had a very low relative abundance, but for compounds **31**, **32** and **35** they were the base peak, the deprotonated ion of the aglycone being also detected (Table 2). The great difference of relative abundances indicates that in compounds **31**, **32** and **35** the acyl bond is easily ruptured, being thus located in a hydroxyl different from position 6 of the hexoses, probably in position 2 of the terminal hexose (Fig. 2), whereas in compounds **10** and **14**–**16** (with a low abundance) the acyl is linked to position 6 of the terminal hexose, as its fragmentation is more difficult. In the MS^3 fragmentation of all the compounds, since they lost substitution at 7, is noticed an ion at *m*/*z* 609 ($[(MH-146)-Acyl]^-$, [kaempferol-3-*O*-di-hexosyl]$^-$) as base peak, besides the losses of −Acyl−162 and/or −Acyl−180 that indicate an interglycosidic linkage 1→2, and the ion of the deprotonated aglycone. This was confirmed by the fragmentation of the ion at *m*/*z* 609, which agreed with the MS^3 fragmentation of **4**/**5** (Scheme 1, data not shown in Table 2). Sinapoyl and feruloyl derivatives also displayed a very abundant ion (sometimes being the base peak), 14 amu higher than the loss of the acyl (−192/−162), and another due to loss of acyl+18 (−224/−194). As expected, *p*-coumaroyl derivatives did not exhibited the loss of acyl−14, being instead observed the loss of water (footnotes of Table 2). These findings indicate that these compounds are cinnamoyl derivatives of **4**/**5** and, according to the above indicated regarding the possible substitution of the acyl, their structures could be tentatively labelled as kaempferol-3-*O*-[6-sinapoyl(2-hexosyl)]hexoside-7-*O*-rhamnoside (**10**), kaempferol-3-*O*-[6-feruloyl(2-hexosyl)]hexoside-7-*O*-rhamnoside (**14**), kaempferol-3-*O*-[6-feruloyl(2-hexosyl)]hexoside-7-*O*-rhamnoside (**15**) isomer, kaempferol-3-*O*-[6-*p*-coumaroyl(2-hexosyl)]hexoside-7-*O*-rhamnoside (**16**), kaempferol-3-*O*-[2-*p*-coumaroyl(2-hexosyl)] hexoside-7-*O*-rhamnoside (**31**), kaempferol-3-*O*-[2-*p*-coumaroyl (2-hexosyl)]hexoside-7-*O*-rhamnoside (**32**) isomer and kaempferol-3-*O*-[2-feruloyl(2-hexosyl)]hexoside-7-*O*-rhamnoside (**35**) (Fig. 2). The isomer compounds must be at the hexose level, as in **4**/**5**.

In the saponified extract, two acyl derivatives not found in the native extract (**A** and **B**) were detected. As so, they could be reaction products or probably formed during the acidification step (after saponification; see Section 2). Similarly to the structure elucidation used, they could be labelled as kaempferol-3-*O*-[6-feruloyl(2-hexosyl)]hexoside-7-*O*-rhamnoside (**A**) isomer and kaempferol-3-*O*-[6-*p*-coumaroyl(2-hexosyl)]hexoside-7-*O*-rhamnoside (**B**) isomer.

3.4. Flavonol-3-O-(DPA, cinnamoyl)glycoside-7-O-rhamnosides

As detailed below and already reported above, the compounds of this group (**24**–**26**, **28**–**30**, **33**, **34** and **36**) are acyl derivatives of the major compounds **4**/**5** (kaempferol-3-*O*-(2-hexosyl) hexoside-7-*O*-rhamnosides), with the exceptions of **29**, an acyl derivative of **12** or **13** (kaempferol-3-*O*-(2-xylosyl)hexoside-7-*O*-rhamnosides), and **36**, an acyl derivative of **20** (kaempferol-3-*O*-glucoside-7-*O*-rhamnosides). Their MS^2 fragmentations presented the typical loss of the glycosylation at position 7 (−146 amu), not always with 100% relative abundance, being sometimes very small or negligible, showing also the losses of the fragments −264 amu and −410 amu (−146−264) characteristic of this group. The ion of deprotonated kaempferol (*m*/*z* 285, with low abundance) was also observed (data not shown in Table 2). The fragmentation of compounds **28**, **30**, **33** and **34** showed further losses of −586/−556 amu (−410−176/−410−146) (Table 2), indicating the presence of an additional feruloyl or *p*-coumaroyl residue. The UV spectrum of compounds **24**–**26** and **36**, the only ones it was possible to see, presented band II (268 nm) with high absorption with respect to that of band I (348 nm). From the exposed above we infer that these flavonoids would be acylated with an acid of mass 282 amu (264+18) and with UV absorption at ~268 nm. Dihydrophaseic acid (DPA), which, together with phaseic acid (PA), is one of the main compounds resulting from the metabolism of abscisic acid (ABA), meets these criteria [6–9]. So, we tentatively propose it as the characteristic acyl group of these compounds. It would have been interesting to observe the UV spectra of the compounds acylated with DPA and cinnamic acid, which probably show absorption bands at 268 and ~320 nm. On the other hand, in all of these compounds (excepting **29** and **36**), the ion at *m*/*z* 609, resulting from the simultaneous loss of the rhamnose at position 7 and of the acids, undergoes a MS fragmentation similar to the MS^3 of **4**/**5** (Scheme 1, data not shown in Table 2); therefore, as mentioned before, they are acylated derivatives of them.

Among the compounds acylated only with DPA (**24**–**26**, **29** and **36**), the allocation of the acylation position of **24**–**26** and **36** is inferred from the low abundance of the ions resulting from the loss of the fragment 264 in MS^2, indicating that the acyl group would be at the hydroxyl at 6 of one of the hexoses, which, as we have herein reported, is difficult to fragment. In turn, in compound **29**, with an abundance of 100%, the acyl group would probably be at position 2 of the terminal sugar.

In the MS^2 of the other compounds (**28**, **30**, **33** and **34**), the ion due to the loss of the cinnamoyl fragment (feruloyl: −176 or *p*-coumaroyl: −146) is not observed, being only detected the loss of −586 or −556 (−410−176/−410−146), also at low abundance. Hence, these acids would be located over the hydroxyl at 6 of the other hexose and these compounds could be considered derivatives of the previous ones by a new acylation. It is not possible to know by MS analysis to which hexoses these acids are linked. In addition, as they can be derivatives of two glycoside isomers (**4**/**5**), the number of possible isomers of the acylated derivatives increases. Given this, these compounds can be tentatively labelled as kaempferol-3-*O*-[6-dihydrophaseoyl(2-hexosyl)]hexoside-7-*O*-rhamnoside isomers (**24**–**26**), kaempferol-3-*O*-[6-dihydropha-

seoyl(2-hexosyl)](6-feruloyl)hexoside-7-*O*-rhamnoside isomers (**28** and **33**), kaempferol-3-*O*-[2-dihydrophaseoyl(2-pentosyl)]hexoside-7-*O*-rhamnoside (**29**), kaempferol-3-*O*-[6-dihydrophaseoyl-(2-hexosyl)](6-*p*-coumaroyl)hexoside-7-*O*-rhamnoside isomers (**30** and **34**) and kaempferol-3-*O*-(6-dihydrophaseoyl)glucoside-7-*O*-rhamnoside (**36**) (Fig. 2).

3.5. Flavonol-3-O-(malonyl)glycoside-7-O-rhamnosides

The MS^2 fragmentation of compounds (**8**, **11** and **22**) was characterized by the loss of 44 amu ($-CO_2$) to yield the base peak, this behaviour being typical of compounds acylated with a dicarboxylic acid. In addition, a combined loss of such fragment and −146 (rhamnosyl) was also observed. The resulting ion ([(M−H)−**44**−146]$^-$) corresponds to the aglycone with the glycosidic fraction at position 3 and the rest of the acyl decarboxylate, which for these compounds would be of 42 amu, corresponds to malonic acid (Table 2).

In the MS^3[(M−H)→(M−H)−**44**−146]$^-$ of compound **8**, besides the ion of the deprotonated aglycone as base peak (*m*/*z* 285, ([kaempferol−H]$^-$), it was observed the loss of −42 amu to yield an abundant ion, indicating a link different from 1→6, probably in position 2 of the terminal hexose, and other combined loss of this fragment with that of an hexose+18 (180 amu) that points to an interglycosidic linkage 1→2. Therefore, this compound could be labelled as kaempferol-3-*O*-[2-malonyl(2-hexosyl)]hexoside-7-*O*-rhamnoside (**8**).

Compound **11**, with the same MS^2 fragmentation of **8**, presented in its MS^3 fragmentation only the ion of the deprotonated aglycone; the absence of the loss of −42 amu and of the ions of the interglycosidic linkage points to 1→6 links. Therefore this compound could be labelled as kaempferol-3-*O*-[6-malonyl(6-hexosyl)]hexoside-7-*O*-rhamnoside (**11**).

Compound **22**, with less 162 amu than **8** and **11**, seems to be a derivative of them with one less hexose. Its MS^2 fragmentation was similar and MS^3 fragmentation was identical to that of **11** (Table 2). Therefore, compound **22** could be labelled as kaempferol-3-*O*-(6-malonyl)hexoside-7-*O*-rhamnoside.

4. Conclusions

Using HPLC-DAD-ESI/MS^n, thirty-seven glycosylated flavonoids (mostly 3-*O*-glycosides) were successfully identified for the first time in the seeds of *L. cicera*. Kaempferol derivatives comprised more than 80% of the compounds found. It was also possible to detect the presence of cinnamoyl, dihydrophaseoyl or malonyl groups acylating the kaempferol glycosides. Advances on the knowledge of compounds with already reported high biological activity in this food matrix was therefore achieved and may contribute to increase the consumption of these grain legumes also by humans, further revaluing this crop.

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CHAPTER 6: ALKALOIDS IN THE VALORIZATION OF EUROPEAN *LUPINUS* SPP. SEEDS CROP

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Alkaloids in the valorization of European *Lupinus* spp. seeds crop

Sara C.Q. Magalhães[a], Fátima Fernandes[b], Ana R.J. Cabrita[a], António J.M. Fonseca[a], Patrícia Valentão[b], Paula B. Andrade[b,*]

[a] REQUIMTE/LAQV, ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal
[b] REQUIMTE/LAQV, Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, R. Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal

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ABSTRACT

In this study, alkaloids from lupin varieties with commercial interest in Europe were identified and quantified. Additionally, the anti-inflammatory and antioxidant potential of some rich-alkaloid lupin extracts was assessed by 5-lipoxygenase (LOX) inhibition and nitric oxide radical (•NO) scavenging assays, respectively. Relationships between extracts activity and those of pure standards were stablished.

Nine alkaloids belonging to quinolizidine, indole and piperidine classes were identified by means of GC-IT/MS and quantified by GC-FID using a validated method. Lupanine was the most abundant alkaloid in white and narrow-leafed lupins (*Lupinus albus* L. and *Lupinus angustifoltus* L., respectively) and sparteine in most yellow lupins (*Lupinus luteus* L.), but their proportions were cultivar-dependent. Gramine, smipine, angustifoline and lupanine derivatives were also identified. Five lupin varieties (Amiga, Estoril, Lumen, Dukat and Mister) were characterized as sweet (<0.5 g alkaloids/kg, dry matter basis) and two of them respected the safety limit imposed by the European health authorities for human consumption (≤0.2 g/kg, dry matter basis). Despite the weak effect on •NO, a dose-dependent response towards LOX was found for all the studied extracts, which followed the order Taper > Estoril > Multitalia-PT > Dukat > Azuro > Multitalia-IT > Nacional.

To our knowledge, the alkaloids composition of some of the varieties, as well as the study of the anti-inflammatory and antioxidant potential of rich-alkaloid lupin extracts are here reported for the first time. The results presented are a source of easily available data for producers, nutritionists and geneticists, and add biological knowledge on a major class of compounds in lupins.

1. Introduction

Lupins (*Lupinus* spp.) are low cost and non-genetic modified legume seeds widely known for their high protein content and overall interesting nutritional value for human food and animal feeding. They provide 30–40% dietary protein, ca. 28% fiber, healthy fatty acids (e.g., linoleic and linolenic acids), vitamins and minerals (Sbihi et al., 2013).

In Europe, white lupins (WL; *Lupinus albus* L.), are usually consumed as snack food (whole seed) and, together with narrow-leafed lupins (NLL; *Lupinus angustifolius* L.), have been gaining interest also as food ingredients (flour) for the manufacture of bread, pasta, biscuits, gluten-free cakes or dairy products (Kohajdova et al., 2011; Villarino et al., 2015). Yellow lupins (YL; *Lupinus luteus* L.) are more appreciated by the intensive livestock industries (Sweetingham and Kingwell, 2008).

Besides nutrients, lupins contain several phytochemicals (e.g. polyphenols, carotenoids, alkaloids, phytosterols) that result from the plant secondary metabolism, being produced in response to diverse biotic and abiotic stresses (e.g. UV radiation, pathogens, herbivores). Phytochemicals are of pharmacological interest as they may positively impact humans and animals' health by providing therapeutic benefits; nonetheless, adverse effects on health are also associated to these compounds, limiting nutrients digestibility and bioavailability and inducing pathological changes in different organ tissues, with impacts on metabolism (Bernhoft, 2010; Khan et al., 2015).

Alkaloids are major phytochemicals in lupins that function as natural agrochemicals (Muzquiz et al., 1994b). However, they deserve extra attention: a safe consumption of lupins presupposes an alkaloid level in the seed as low as possible (Lucas et al., 2015). Acute toxicity of lupin alkaloids in humans comprises neurologi-

* Corresponding author.
E-mail address: pandrade@ff.up.pt (P.B. Andrade).

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cal, cardiovascular and gastrointestinal disturbances, children, in particular, being more sensitive (Koleva et al., 2012). In feedstuffs, lupins bitter taste, highly related to the seed alkaloids content (Dupont et al., 1994), may decrease diet palatability, affecting feed intake and body weight gain (Pastuszewska et al., 2001; Pilegaard and Gry, 2009). The safety limit fixed by the health authorities of UK, France, Australia and New Zealand for the total amount of alkaloids in lupin flours and derived products is of 0.2 g/kg dry matter (DM; Pilegaard and Gry, 2009).

Although alkaloids may be toxic when ingested at high concentrations, several biological properties were already described for rich-alkaloid lupin extracts, such as antimutagenic, antibacterial, antifungal and anticancer, a topic recently reviewed by Khan et al. (2015). As far as we are aware, the anti-inflammatory and antioxidant potential of these lupins secondary compounds has not been studied yet.

The present work aimed, firstly, at determining the alkaloids composition of several European lupin varieties of commercial interest by means of advisable chromatographic techniques. Furthermore, aiming to broaden the knowledge on the biological potential of these matrices, the anti-inflammatory and antioxidant potential of rich-alkaloid lupin extracts (at non-toxic concentrations for consumption) was also screened in a cell-free system, by evaluating the 5-lipoxygenase (LOX) inhibitory capacity and the nitric oxide radical (•NO) scavenging activity, respectively. In an attempt to relate the effect observed in the extracts with their composition in alkaloids, pure compounds were also evaluated individually.

As the 68th United Nations General Assembly declared 2016 as the International Year of Pulses (United Nations, 2014), we consider of interest to study a major group of phytochemicals in lupins from a nutritional and pharmacological perspective.

2. Material and methods

2.1. Materials and reagents

(-)-Sparteine (97%), angustifoline (>95%) and lupanine (>95%) were purchased from ChemFaces (Wuhan, Hubei, China). (-)-Lupinine (only for qualitative purposes, as indicated by the supplier) and quercetin were obtained from Extrasynthese (Lyon Nord, France). Gramine (99%), trichloroacetic acid, dichloromethane, the *n*-alkane series (C8-C40), potassium dihydrogen phosphate, sulphanilamide, lipoxidase from *Glycine max* (L.) Merr. (type V-S; EC 1.13.11.12), cis-9,12-octadecadienoic acid (≥99.0%) and ethanol were obtained from Sigma (St. Louis, MO). Sodium hydroxide was purchased from VWR (Radnor, PA). *N*-(1-Naphthyl)-ethylenediamine dihydrochloride was obtained from Acros Organics (Waltham, MA,). Sodium nitroprusside dihydrate (SNP) was from Riedel-de Haën (St. Louis, MO). Fosforic acid and di-sodium hydrogen phosphate dihydrate were acquired from Merck (Darmstadt, Germany). Water was treated in a Milli-Q (Millipore, Bedford, MA) water purification system.

2.2. Sampling

Eleven varieties (included in the European Plant Variety Database (PVD, 2015)) and one Portuguese ecotype of lupins, corresponding to mature raw seeds of 5 white lupins (*L. albus*), 2 narrow-leafed lupins (*L. angustifolius*) and 5 yellow lupins (*L. luteus*) (Table 1), were analyzed. Seeds were dried in a forced-air oven (65 °C, 24 h) and grounded (1 mm). Dry matter content of lupin flours was determined after drying the powdered samples at 103 °C overnight (AOAC, 2000).

Table 1
Characterization of the studied lupins samples.

Material	Sample code	Sample origin	Breeder country[a]
White lupin			
Amiga	WL-A	France	Czech Republic, France
Lumen	WL-L	France	France
Estoril	WL-E	Portugal	Portugal
Multitalia-PT	WL-M-PT	Portugal	Italy
Multitalia-IT	WL-M-IT	Italy	Italy
Yellow lupin			
Dukat	YL-D	Poland	Poland
Taper	YL-T	Poland	Poland
Mister-PT	YL-M-PT	Portugal	Poland
Mister-PL	YL-M-PL	Poland	Poland
Nacional	YL-N	Portugal	Portugal
Narrow-leafed lupin			
Azuro	NLL-A	Portugal	Denmark
Sonet	NLL-S	Poland	Denmark, Poland

[a] According to the European Plant Variety Database (PVD, 2015).

2.3. Alkaloids extraction

Alkaloids were extracted as previously described by Muzquiz et al. (1994a) and Gresta et al. (2010), with slight modifications. Briefly, 2.0 g of seed flour (1 mm) were added to 20 mL of trichloroacetic acid (5%, w/v), homogenized in a magnetic stirrer for 30 min at 400 rpm and then centrifuged at 4000 rpm for 15 min. The extraction procedure was repeated twice and the supernatants were decanted while the solid residue was discarded. 4 mL of 10 M sodium hydroxide were added to the supernatant. The alkaloids fraction was separated with dichloromethane (3 × 20 mL). The resulting dichloromethane extract was evaporated to dryness under reduced pressure (40 °C) and stored at −20 °C protected from light, until analysis. The yields (g extract/kg seed DM) obtained were 2.64 for WL var. Estoril (WL-E), 1.63 for WL var. Amiga (WL-A), 7 1.64 for WL var. Multitalia from Italy (WL-M-IT), 22.19 for WL var. Multitalia from Portugal (WL-M-PT), 8.59 for WL var. Lumen (WL-L), 23.09 for YL ecotype Nacional (YL-N), 0.37 for YL var. Mister from Portugal (YL-M-PT), 1.02 for YL var. Mister from Poland (YL-M-PL), 2.49 for YL var. Taper (YL-T), 0.29 for YL var. Dukat (YL-D), 33.11 for NLL var. Azuro (NLL-A) and 3.00 for NLL-Sonet (NLL-S).

2.4. GC-IT/MS qualitative analysis of alkaloids

Rich-alkaloids extracts were redissolved with dichloromethane, filtered (0.45 μm) and analyzed by GC-IT/MS. Stock solutions of alkaloids were prepared individually in dichloromethane, filtered (0.45 μm) and kept at −20 °C until analysis.

GC-IT/MS analysis was performed following a previously established method (Gresta et al., 2010). Samples extracts (1 μL) were analyzed using a Varian CP-3800 gas chromatograph (USA) equipped with a Varian Saturn 4000 ion trap mass detector (USA) and a Saturn GC–MS workstation software version 6.8. Analysis were carried out using a capillary column VF-5 ms (30 m × 0.25 mm × 0.25 μm) from Varian. The oven temperature was set at 150 °C for 1 min, then increased at a rate of 5 °C/min to 235 °C (held for 15 min). High purity helium C-60 (Gasin, Portugal) was the carrier gas at a constant flow rate of 1.0 mL/min. The injector port was heated to 240 °C and the injections performed in a split mode, with a ratio of 1/10. All mass spectra were acquired in electron impact (EI) mode. Ionization was maintained off during the first 3 min to avoid solvent overloading. The detection was performed using an Ion Trap detector set as follows: the transfer line, manifold and trap temperatures were 280, 50 and 180 °C, respectively. The mass range was 50–1000 *m/z*, with a scan rate of 6 scan/s. The emission current was 50 μA, and the electron multiplier was

set in relative mode to auto-tune procedure. The maximum ionization time was 25,000 μs, with an ionization storage level of 35 *m/z*. Analysis was performed in Full Scan mode.

The compounds were identified by comparison of their mass spectra with those from pure standards analyzed under the same conditions, with data of the literature (Wink et al., 1995) and with NIST 05 MS Library Database (WebBook, 2015). In addition, the retention indices (RI) were experimentally calculated using the homologous series of *n*-alkanes, and the values were compared with those reported in the literature for GC columns with 5%-phenyl-95%-dimethylpolysiloxane (Wink et al., 1995).

2.5. Alkaloids quantification by GC-FID

Quantitative analysis of the dichloromethane extracts was performed using a Finnigan Focus GC (Thermo Electron Corporation) equipped with a FID and a VF-5 ms (30 m × 0.25 mm × 0.25 μm) column (Varian). The injector and detector temperatures were 240 and 250 °C, respectively. Elution conditions used were the same described above for GC-IT/MS analysis. Each lupin extract (1 μL) was injected in triplicate.

The quantification of each alkaloid present in the extracts was achieved from the calibration curves of the respective standard analyzed under the same conditions. Since standards of some identified compounds were not commercially available or their degree of purity was not adequate for quantitative purposes, alkaloids quantification was achieved as follows: gramine, sparteine, angustifoline and lupanine were quantified as themselves, whereas lupinine, smipine, α-isolupanine, 11,12-dehydrolupanine, and 13-hydroxylupanine were quantified as lupanine.

2.6. GC-FID method validation

2.6.1. Linearity

The linearity range of the method was assessed by building calibration curves using, at least, six different concentration levels of the analytes, according to the range of concentrations present in the samples.

2.6.2. Limits of detection and of quantification

Limit of detection (LOD) and limit of quantification (LOQ) were determined from calibration curve data, following the formula:

$$LOD = (3.3xSD)/b$$

$$LOQ = (10xSD)/b \quad ,$$

where *SD* is the residual standard deviation of the linear regression, and *b* is the slope of the regression line.

2.6.3. Precision and recovery tests

The repeatability and reproducibility of the method were calculated in terms of intra-day and inter-day precision, respectively. Repeatability was performed by injecting the same lupin sample, by the same analyst, 5 times in the same day, whereas, reproducibility was performed by injecting that same sample in triplicate during 5 consecutive days. Final results were expressed as coefficient of variation (CV, %) (Table 2). Recovery tests were performed by spiking a lupin sample with three different concentrations (low, medium, high) of standards.

2.7. 5-LOX inhibition

The inhibitory effect on LOX was assessed in 96-well plates, using a procedure previously reported (Pereira et al., 2015). Briefly, the blank was measured in a reaction mixture with 20 μL of each extract/compound, 200 μL of phosphate buffer (pH 9.0) and 20 μL of LOX 100 U. Soybean LOX (5-, 12-, and 15-) is widely accepted to model human due to difficulties in obtaining human LOX for bioassays and due to the high catalytic domain similarity between plant and mammalian LOX (Porta and Rocha-Sosa, 2002; Skrzypczak-Jankun et al., 2003). After 5 min pre-incubation at room temperature, the reaction was started by addition of 20 μL of substrate (C18:2*n*-6c) at 4.18 mM in ethanol. The reaction was monitored at 234 nm on a Synergy™ HT plate reader (Biotek Instruments, Winooski, USA) operated by Gen5 Software, for 3 min. Quercetin was used as positive control. Three experiments were performed in triplicate.

2.8. •NO scavenging

Antiradical activity of the extracts was assessed spectrophotometrically in a 96-well plate reader (Multiskan Ascent, Thermo Lab Systems), according to the described procedure (Vrchovska et al., 2007). The reaction mixtures in the sample wells consisted of extract/compound and sodium nitroprusside and plates were incubated for 60 min under light exposure. Griess reagent was then added and the mixture was incubated at room temperature for 10 min, in the dark. Absorbance was determined at 562 nm. Three experiments were performed in triplicate.

2.9. Statistical analysis

The statistical analyses involving the experimental data of alkaloids profiling were performed using SPSS (version 22.0, IBM SPSS Statistics, USA). Mean values were compared by one-way analysis of variance (one-way ANOVA). When ANOVA indicated a significant difference ($P < 0.05$), Tukey's HSD test was performed. Principal component analysis (PCA), performed based on normalized data, was applied for reducing the number of variables (ten variables corresponding to the total alkaloids content and to each quantified (>LOQ) alkaloid: lupinine, smipine, gramine, sparteine, angustifoline, α-isolupanine, lupanine, 11,12-dehydrolupanine and 13-hydroxylupanine) to a smaller number of the new derived variables (principal components, PCs) that adequately summarize the original information, i.e., the alkaloids composition of studied lupin samples. PCA method shows similarities between samples projected on a plan and makes it possible to identify which variables determine these similarities and in what way.

3. Results and discussion

3.1. Alkaloids profile

With alkaloids as major phytochemicals in lupins, it is important for producers, nutritionists and geneticists to know the accurate composition of alkaloids present in lupin varieties of commercial interest.

Nine alkaloids were identified in lupin dichloromethane extracts, namely, lupinine, sparteine, angustifoline, α-isolupanine, lupanine, 11,12-dehydrolupanine and 13-hydroxylupanine (all quinolizidine alkaloids, QA), smipine (piperidine alkaloid) and gramine (indole alkaloid; Table 3). The alkaloids profile of a representative sample of WL, YL and NLL is shown in Fig. 1A–C, respectively. Lupinine, gramine and sparteine were the alkaloids detected in all YL extracts. Angustifoline, lupanine and α-isolupanine were alkaloids common to WL and NLL. Additionally, WL also presented in their composition lupinine, smipine, 11,12-dehydrolupanine and 13-hydrolupanine. The obtained profile for each lupin species was in agreement with earlier studies (Aniszewski, 2015; Reinhard et al., 2006; Wink et al., 1995).

Table 2
Linearity, detection and quantification limits, repeatability, reproducibility and recovery of alkaloids with the employed analytical conditions.

Alkaloids	Regression equation	R^2	Linearity (mg/mL)	LOD (mg/mL)	LOQ (mg/mL)	Repeatability (CV, %)	Reproducibility (CV, %)	Recovery[a] (%)
Gramine	$y = 3.5 \times 10^6x + 20931.9$	0.9971	$7.8 \times 10^{-3} - 2.0$	1.4×10^{-3}	4.4×10^{-3}	9.5	10.8	106/106/96
Sparteine	$y = 1.7 \times 10^6x - 20452.0$	0.9990	$2.0 \times 10^{-3} - 2.0$	1.2×10^{-5}	3.6×10^{-5}	6.8	11.2	103/130/77
Angustifoline	$y = 3.5 \times 10^6x - 37971.0$	0.9989	$3.9 \times 10^{-3} - 1.0$	0.7×10^{-3}	2.0×10^{-3}	7.6	7.4	99/87/87
Lupanine	$y = 2.1 \times 10^6x - 1817.2$	0.9981	$9.7 \times 10^{-4} - 5.0$	8.5×10^{-5}	2.6×10^{-4}	7.2	5.2	111/98/109

CV, coefficient of variation; LOD, limit of detection; LOQ, limit of quantification.
[a] Recovery of the compound at low/medium/high concentrations, respectively.

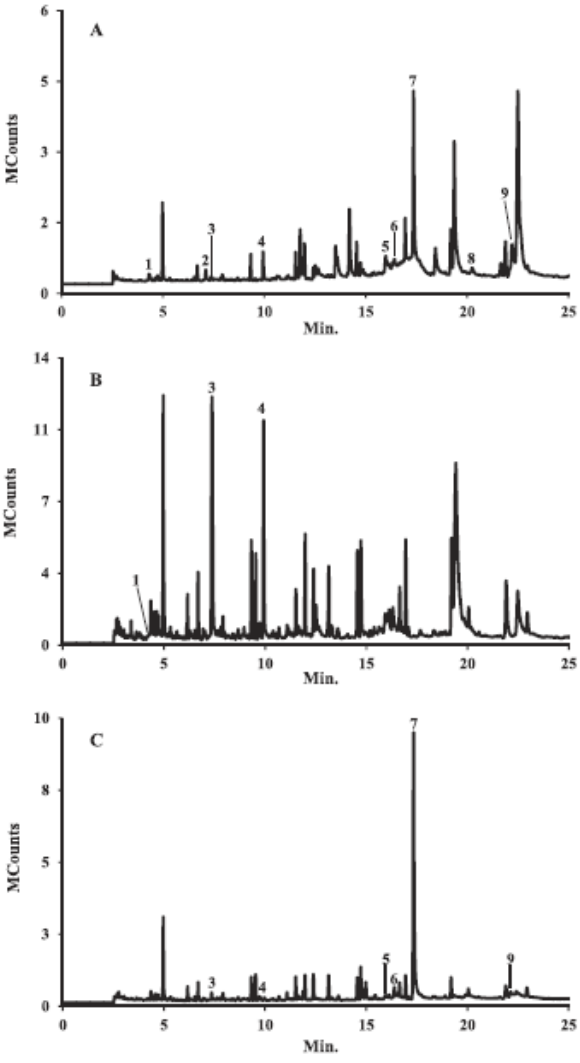

Fig. 1. GC–MS chromatograms of (A) *L. albus* var. Estoril, (B) *L. luteus* var. Taper, and (C) *L. angustifolius* var. Sonet. **(1)** Lupinine; **(2)** Smipine; **(3)** Gramine; **(4)** Sparteine; **(5)** Angustifoline; **(6)** α-Isolupanine; **(7)** Lupanine; **(8)** 11,12-Dehydrolupanine; **(9)** 13-Hydroxylupanine.

According to Aniszewski (2015), albine, angustifoline, lupanine and sparteine are the main alkaloids in *L. albus* seeds, lupinine and sparteine in those of *L. luteus* and, finally, angustifoline in *L. angustifolius*. Indeed, QA (bi-, tri and tetracyclic), which derive from the amino acid lysine, characterize the Fabaceae family (Aniszewski, 2015; Bruneton, 2009) and are ubiquitous in lupin species (Wink et al., 1995). Despite albine, multiflorine and esters of 13-hydroxylupanine (e.g., 13-angeloyloxylupanine and 13-tigloyloxylupanine) have already been described in seeds of *L. albus* (Coïsson et al., 2011; Muzquiz et al., 1994a; Sanchez et al., 2005), they were not detected herein.

The calibration plots of gramine, sparteine, angustifoline and lupanine exhibited correlation coefficient values higher than 0.99 (Table 2). Calculated LOD and LOQ are shown in Table 2. Alkaloids recovery ranged between 99 and 111%, 87–130%, and 77–109% for low, medium, and high concentrations, respectively. Recovery values above 100% result from the matrix potentiation effect (Matuszewski et al., 2003). The CV obtained for intra-day and inter-day precision were lower than 10% and 12%, respectively (Table 2), meaning the method showed good repeatability and a satisfactory reproducibility.

Lupanine was the major alkaloid in samples of WL and NLL (ca. 0.2–46.2 and 0.6–20.6 g/kg DM, respectively) whereas sparteine was the most abundant compound in all YL samples (0.1-7.9 g/kg DM), excluding in YL-T which showed higher levels of gramine than sparteine (ca. 0.6 and 0.2 g/kg DM, respectively). Indeed, tetracyclic lupanine and sparteine are the most representative alkaloids in seeds of lupins (Aniszewski, 2015). Lupinine was present at trace amounts in all YL, except in YL-N (ca. 2.4 g lupinine/kg DM). In WL samples, angustifoline was highest ($P < 0.001$) for WL-M-IT (ca. 0.8 g/kg DM) followed by WL-M-PT (0.3 g/kg DM) whereas in NLL samples, angustifoline was present at significantly higher levels in NLL-A than in NLL-S ($P < 0.001$; ca. 3.8 and 0.1 g/kg DM, respectively). Lupanine derivatives, namely 11,12-dehydrolupanine and 13-hydroxylupanine, were found at low amounts (≤0.1 g/kg DM) in all WL, except in WL-M-IT (0.9 and 4.7 g/kg DM, respectively; Table 3). Contrarily to lupanine, their derivatives are usually present as minor alkaloids (up to 0.1 g/kg; Boschin et al., 2008; Muzquiz et al., 1994a). Apparently, 13-hydroxylupanine appears at high levels, together with angustifoline, when it comes to rich-alkaloid accessions (Boschin et al., 2008; Sanchez et al., 2005) a fact that justifies the quantitative profile observed for WL-M-IT, the lupin sample with the greatest levels of alkaloids.

Contrarily to QA, indole alkaloids (deriving from the amino acid tryptophan), such as gramine and its derivatives, are not usually detected in lupins (Wink et al., 1995). In the present work, however, YL-N and YL-T presented 0.3 and 0.6 g gramine/kg DM, respectively, whereas all the other samples presented trace amounts of it (Table 3). The level of this alkaloid in YL-T agrees with findings of Wiatr (1999), referring that gramine in Polish YL can vary between 0.8 and 1.0 g/kg. There is evidence that high gramine content in YL is related to increased resistance against aphids, as this is considered to be the most toxic alkaloid affecting these insects (Ridsdill-Smith et al., 2004).

Although lupinine is reported to be abundant in YL, representing 60% of total alkaloids (Wink et al., 1995), it was found at high levels only in YL-N (2.4 g/kg DM), which was also the lupin sample with the highest sparteine level, being therefore the richest YL in terms of alkaloids. As previously stated, Nacional is an ecotype. Ecotypes, also referred to as old varieties (Boschin et al., 2008),

Table 3
Alkaloids in the seeds of white (WL), yellow (YL) and narrow-leafed (NLL) lupins (g/kg, dry matter basis).[a]

	Alkaloid	RI	RI	ID	Identification ions	WL-					YL-					NLL-	
		Exp	Lit			E	A	M-IT	M-PT	L	N	M-PT	M-PL	T	D	A	S
	Quinolizidine Bicyclic																
1	Lupinine	1450	1420	S	55/97/110/138/152/168	0.04 (0.00)[a]	0.01 (0.00)[a]	nq	0.03 (0.00)[a]	0.05 (0.00)[a]	2.42 (0.23)[b]	0.00[a]	0.00[a]	0.00[a]	0.00[a]	nd	nd
	Tricyclic																
5	Angustifoline	2152	2083	S	55/64/112/150/193	0.01 (0.00)[a]	0.01 (0.00)[a]	0.75 (0.01)[b]	0.26 (0.01)[c]	0.04 (0.00)[a]	nd	nd	nd	nd	nd	3.83 (0.14)[d]	0.09 (0.00)[a]
	Tetracyclic																
4	Sparteine	1812	1785	S	98/137/193/235	nq	nq	nq	nq	nq	7.89 (0.05)[a]	0.28 (0.00)[b]	0.71 (0.00)[c]	0.22 (0.00)[d]	0.12 (0.00)[f]	nd	0.01 (0.00)[f]
6	α-Isolupanine	2178	2105	T	55/98/136/149/219/248	0.01 (0.00)[a]	0.01 (0.00)[a]	0.25 (0.00)[b]	0.08 (0.00)[c]	0.02 (0.00)[a]	nd	nd	nd	nd	nd	0.55 (0.01)[d]	0.04 (0.01)[e]
7	Lupanine	2232	2165	S	55/98/136/148/248	0.20 (0.00)[a]	0.16 (0.00)[a]	46.24 (0.62)[b]	12.19 (1.16)[c]	0.28 (0.03)[a]	nd	nd	nd	nd	nd	20.57 (0.36)[d]	0.55 (0.02)[a]
8	11,12-Dehydrolupanine	2249	2190	T	55/82/134/148/246	0.11 (0.00)[a]	0.01 (0.00)[a]	0.87 (0.00)[b]	nq	0.06 (0.01)[a]	nd	nd	nd	0.28 (0.01)[c]	nd	nd	nd
9	13-Hydroxylupanine	2467	2400	T	55/134/152/165/246/264	0.04 (0.00)[a]	0.00[a]	4.71 (0.14)[b]	nq	0.02 (0.00)[a]	nd	nd	nd	0.01 (0.00)[a]	nd	nq	nq
	Piperidine Bicyclic																
2	Sninine	1648	1580	T	81/99/109/151	0.03 (0.00)[a]	0.01 (0.00)[b]	nd	0.02 (0.00)[c]	0.03 (0.00)[a]	nd	nd	nd	nd	nd	nd	nd
	Indole Bicyclic																
3	Gramine	1665	1622	S	77/102/130/175	nq	nq	nq	nq	nq	0.33 (0.01)[a]	0.01 (0.00)[b]	0.00[b]	0.56 (0.00)[c]	0.00[b]	nq	nq
	TOTAL					0.44 (0.01)[a]	0.22 (0.00)[a]	52.38 (1.38)[b]	12.58 (1.16)[c]	0.49 (0.04)[a]	10.63 (0.18)[c]	0.28 (0.00)[a]	0.71 (0.00)[a]	1.06 (0.00)[a]	0.13 (0.00)a	24.95 (0.43)[d]	0.69 (0.02)[a]

RI Exp – Retention Indices obtained in this experiment, calculated according to Kovats (1958), as follows $100x\left(\left(tx-tn\right)/\left(tn+1-tn\right)\right)+n$, where tx is the retention time of the compound, tn is the retention time of the preceding n-alkane, tn+1 is the retention time of the following n-alkane and n is the preceding n-alkane; RI Lit – Retention Indices described in the literature (Wink et al., 1995).
ID – Identification: S: identified by comparison with authentic standard; T: tentatively identified by comparison of mass spectrum and RI with the literature (Wink et al., 1995) and NIST 05 Library Database (WebBook, 2015).
Samples identity as in Table 1.
nd, not detected; nq, not quantified.
Different superscript letters in the same row indicate significant differences ($P<0.001$).
[a] Results are expressed as mean (standard error of the mean) of three determinations.

are populations well adapted to specific environmental conditions. As so, high amounts of alkaloids in ecotypes (Boschin et al., 2008; Muzquiz et al., 1994a) play a chemical defense role, contributing to their increased hardiness against pests and diseases (Sweetingham and Kingwell, 2008). This is, in fact, the dual character associated to alkaloids: while protecting crops life and maintaining a strong biological and ecological order in nature, they are also able to kill or weaken life in other cells and units with their toxic effects (Aniszewski, 2015; Bruneton, 2009).

The alkaloid content of Multitalia samples and, in particular that of lupanine, was quite higher than previously described for this variety (ca. 12.2–46.2 vs. 1.1–1.5 g/kg DM; Calabrò et al., 2015; Gresta et al., 2010). Multitalia is a well-known Italian variety, reported to contain no or low amounts of alkaloids. Boschin et al. (2008) and Annicchiarico et al. (2014) stated that high alkaloid values found in alleged "low alkaloid" varieties appear to result of pollen transference from bitter material during seed multiplication, capable of causing a genetic shift that is perpetuated through the years, not necessarily reflecting the intrinsic genetic characteristics of the varieties. Indeed, despite WL being predominantly self-pollinating, outcrossing due to the activity of bumble bees ranges from 5 to 10% (Huyghe, 1997). Based on the percentage of bitter seeds in the original seed batch (e.g. 3%), on the number of multiplication generations and on the selective advantage of the bitter plants (e.g. 1.2), the percentage of bitter seeds can reach 9% after 4 multiplications and 25% after 8 multiplications, if 10% of outcrossing is considered (Huyghe, 1997). This fact could help explaining the significant quantitative differences ($P<0.001$) found between both Multitalia samples herein studied (once they were multiplied in different countries). Nonetheless, it is noteworthy that intraspecific variability exist in *L. albus* for most morphological characters, for seed size and seed composition as a result of diverse soil and climatic conditions and biotic aspects (pests; Huyghe, 1997).

Also both Mister YL presented identical profile but quantitative differences ($P<0.001$) in terms of alkaloids: sparteine was present at higher levels in YL-M-PL than in YL-M-PT (ca. 0.7 and 0.3 g/kg DM, respectively). As these samples grew in quite distinct countries (Poland and Portugal), differences observed may suggest a production site-effect. Although we do not know the edapho-climatic conditions that occurred during the growth of none of the analyzed samples, previous studies on the effects of different environmental conditions on lupins alkaloids suggest that higher amounts of sparteine in YL-M-PL may be related to the adequate phosphorus content (Gremigni et al., 1999) and higher pH of Polish soils (Jansen et al., 2012) as well as with the lower temperatures usually observed in Poland (Boschin et al., 2008). Also, terminal drought stress to which lupins are subjected in Mediterranean areas appears to decrease their alkaloids content (Annicchiarico et al., 2014; Christiansen et al., 1997), agreeing with the 2.5-fold lower alkaloid amounts of YL-M-PT compared with YL-M-PL.

Concerning to the total alkaloid content of the lupin samples, they all presented similar values ($P>0.05$; 0.1–1.1 g/kg DM), except WL-M-PT, WL-M-IT, YL-N and NLL-A, whose values ranged between ca. 10.6 and 52.4 g/kg DM. Because Poland is a major European lupin producer, autochthonous varieties are frequently characterized in terms of alkaloids. The values herein obtained for the total alkaloids content of Polish varieties grown in Poland (YL-M-PL, YL-T, YL-D and NLL-S) were similar to those previously reported (Kordan et al., 2012; Sujak et al., 2006). Low levels of QA in WL-A had also been described (Coïsson et al., 2011), agreeing with our findings. To the best of our knowledge, this study reports for the first time the alkaloids composition of WL-E, WL-L, YL-N and NLL-A, as well as, of those of Multitalia and Mister when grown in Portugal (WL-M-PT and YL-M-PT, respectively).

PCA was applied to identify patterns in our data set that highlight similarities and differences between lupin samples, in terms

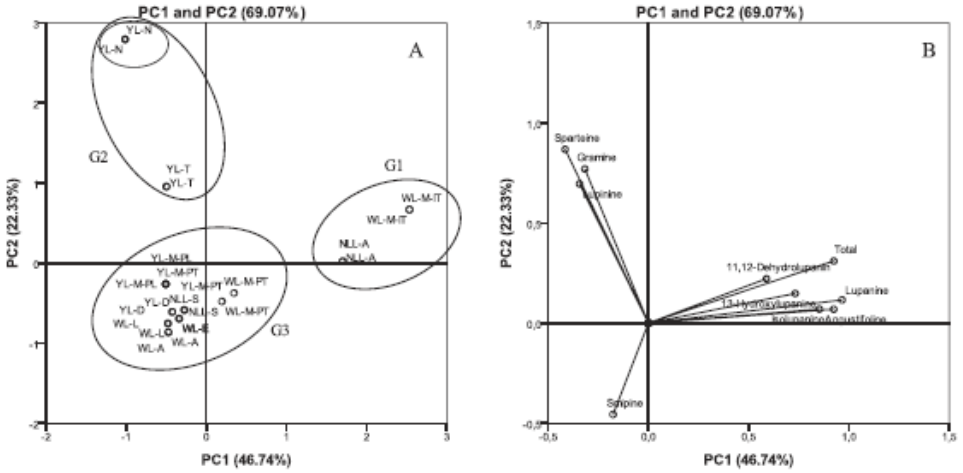

Fig. 2. Projection of lupin samples (**A**) (variables: white lupin (WL) var. Estoril (WL-E); WL var. Amiga (WL-A); WL var. Multitalia-IT (WL-M-IT); WL var. Multitalia-PT (WL-M-PT); yellow lupin (YL) ecotype Nacional (YL-N); YL var. Mister-PT (YL-M-PT); YL var. Multitalia-PL (YL-M-PL); YL var. Dukat (YL-D); YL var. Taper (YL-T); narrow-leafed lupin (NLL) var. Azuro (NLL-A); NLL var. Sonet (NLL-S)) and loadings (**B**) by alkaloids and total alkaloids content into the plane composed by the principal components PC1 and PC2 containing 69.07% of the total variance.

of their total and individual alkaloids composition. Two PCs were retained, corresponding to eigenvalues >1, and explained 69.07% of total data variability. The first one (PC1) represented 46.74% of the variation and was associated with total alkaloids content, and with the compounds angustifoline, lupanine and lupanine's derivatives, whereas component two (PC2), responsible for 22.33% of the variation, was mainly represented by lupinine, sparteine and gramine (Fig. 2). As shown in Fig. 2A, three groups can be clearly distinguished. One group (G1) includes WL-M-IT and NLL-A for presenting the highest levels of angustifoline, lupanine, lupanine derivatives and total alkaloids. YL-N and YL-T constituted group 2 (G2), appearing in the negative plan of PC1 and positive plan of PC2 mainly given their higher contents in gramine and in total alkaloid ($P < 0.001$) comparing to the other YL samples. Within G2, YL-N can constitute a sub-group; indeed, relatively to YL-T, besides with greater ($P < 0.001$) amount of total alkaloids, YL-N also presents higher ($P < 0.001$) levels of lupinine and sparteine. All the other lupin samples formed group 3 (G3). In common, they present negligible or no amounts of lupinine, angustifoline and lupanine derivatives. Within WL varieties of G2, higher amounts of lupanine and angustifoline, and therefore of total alkaloids content, in WL-M-PT than in WL-E, WL-A and WL-L are responsible for its position in the positive plan of PC1.

3.2. Interest for consumption

Among all studied samples, only three varieties of WL (WL-E, WL-A and WL-L) and two of YL (YL-D and YL-M-PT) presented a total alkaloid content below 0.5 g/kg DM, being considered as sweet (Pilegaard and Gry, 2009). Still, only two (WL-A and YL-D) respected the safety limit currently fixed by the health authorities of UK, France, Australia and New Zealand for the total amount of alkaloids allowed in lupin flours and derived products (≤ 0.2 g/kg DM; Pilegaard and Gry, 2009) and could, therefore, be safely added in the diet of human consumers as raw seeds. Studies on rats suggest that an intake of 0.035-0.125 mg of lupin alkaloids/kg body weight per day is not of safety concern for humans (Koleva et al., 2012), resulting for a 60 kg person in a daily consumption of at most 11–38 g (DM basis) of raw modern lupin seeds. In order not to compete with the primary use as food ingredients, sweet lupins (mainly WL) should be considered for animal feeding only if the seed caliber is not of interest for human consumption. On the other hand, the sweet YL, commonly more intended for feed purposes, could be included in diets for animals. For example, the high protein requirements of salmonid and prawn diets were identified as a market niche for YL in extruded diets (Sweetingham and Kingwell, 2008).

For the bitter varieties found in the present work, and especially for those containing very high levels of alkaloids (>10.0 g/kg DM), it would be recommended a debittering process in order not to compromise intake, to ensure a safer consumption and to allow increasing lupins dietary levels in humans or animals diets. Detoxification of lupin seeds through acidic (0.5% HCl) or alkaline (0.5% $NaHCO_3$) treatments (Jiménez-Martínez et al., 2001; Tadele, 2015), as well as germination of lupin seeds for maximum 3 days (Sanchez et al., 2005), are good examples of debittering processes.

3.3. Bioactivity

Of the 12 lupin samples characterized for the alkaloids profile, eight rich-alkaloid extracts were chosen also to evaluate 5-LOX inhibition and •NO scavenging activity, in cell-free systems, based on their high and low alkaloids content and profile (Table 3).

Inflammation is now an important theme in biomedical research, once it plays a key role in several diseases, such as arthritis, diabetes, heart disease, Alzheimer's and Parkinson's diseases, allergies, asthma, cancer, among others. Due to the increased prevalence and incidence of these diseases worldwide, the need for new molecules with anti-inflammatory properties is urgent (Adebayo et al., 2015). The mechanisms of action of many promising anti-inflammatory compounds are thought to be via their free radical scavenging activities or via the inhibition of pro-inflammatory enzymes, such as cyclooxygenases (COX) and LOX, in the inflammatory cascades (Sadik et al., 2003). LOX are lipid-peroxidizing enzymes involved in the biosynthesis of leukotriene from arachidonic acid, mediators of inflammatory and allergic reactions. These enzymes catalyze the addition of molecular oxygen to unsaturated fatty acids like linoleic and arachidonic acids (Porta and Rocha-Sosa, 2002). So, extracts or compounds from natural sources inhibiting the pro-inflammatory activities of these enzymes may constitute promising anti-inflammatory drugs (Adebayo et al., 2015).

Table 4
IC_{25} (mg/mL) values for LOX inhibition by white, yellow and narrow-leafed lupins rich-alkaloid extracts and by pure alkaloid standards.

Samples	IC_{25} values
WL-	
Estoril	0.136
Multitalia-IT	0.525
Multitalia-PT	0.229
YL-	
Nacional	0.766
Taper	0.104
Dukat	0.341
NLL-	
Azuro	0.416
Sonet	>0.354
Standards	
Gramine	0.119
Spartein	>0.077
Lupanine	>0.077
Angustifoline	>0.077
Positive control	
Quercetin	0.00051

Although many studies have focused on the LOX activity originated from lupins (Jacobo-Velazquez et al., 2010; Olias and Valle, 1988; Yoshie-Stark and Wasche, 2004), only few studies have documented the LOX inhibitory potential of this vegetable material. *In vitro* anti-inflammatory properties of protein hydrolysates from seeds of *L. angustifolius* were previously investigated using a macrophage model: lupin protein hydrolysates significantly inhibited the NO production (inflammatory mediator) by phorbol 12-myristate 13-acetate (PMA)-stimulated macrophages (Millan-Linares et al., 2014). Gamarra-Castillo et al. (2006) reported very promising anti-inflammatory activity of aqueous extracts from *Lupinus mutabilis* L. in *in vivo* assays. In a different approach, the reduction of the activity of lupins endogenous LOX by (a) thermal inactivation (Stephany et al., 2016a,b) and (b) the inhibitory effect exerted by lupins native phenolic compounds (Czubinski et al., 2012), has been referred as a highly important process of preservation of the seeds organoleptic and nutritional characteristics. Indeed, LOX-catalyzed degradation of polyunsaturated fatty acids is supposed to be a major cause of undesirable off-flavour development in legumes (Stephany et al., 2015); the resulting hydroperoxide products can lead to reactions of formed peroxyl radical complexes with vitamins, pigments, proteins and polyphenols, further decreasing overall quality of lupins (Czubinski et al., 2012).

The rich-alkaloid lupin extracts of WL-M-PT (0.623–0.039 mg of dried extract/mL), WL-M-IT (1.031–0.064 mg of dried extract/mL), WL-E (0.302–0.019 mg of dried extract/mL), YL-N (1.940–0.121 mg of dried extract/mL), YL-D (0.415–0.026 mg of dried extract/mL), YL-T (0.331–0.021 mg of dried extract/mL), NLL-A (1.089–0.068 mg of dried extract/mL) and NLL-S (0.354–0.022 mg of dried extract/mL) exhibited a concentration-dependent LOX inhibitory capacity (Fig. 3A). According to the effect observed they were ordered as follows: YL-T > WL-E > WL-M-PT > YL-D > NLL-A > WL-M-IT > YL-N (Table 4). Due to solubility issues, it was not possible to determine the IC_{25} value of NLL-S: 18% inhibition was noticed for the maximum concentration tested (0.354 mg of dried extract/mL). The rich-alkaloids extracts studied herein revealed, therefore, a moderate LOX-inhibitory potential. There was not a direct relation between extracts activity and their total alkaloid content. In fact, the most potent extract (of YL-T) was not the richest one. Also, WL-M-IT extract, containing the highest alkaloid levels (Table 3), was one of the least active.

Pure compounds also inhibited LOX in a concentration-dependent manner (Fig. 3B), gramine displaying the strongest effect (Table 4; Fig. 3B). Again, as spartein, lupanine and angustifoline revealed low solubility in the phosphate buffer used in the assay, the highest concentration tested was 0.077 mg/mL, which corresponded to 13, 18 and 23% inhibition, respectively. Both lupin extracts and pure standards revealed lower inhibitory capacity than quercetin, the positive control used. Relating the extracts activity with that of pure compounds, one can see that YL-T LOX inhibition may be greatly attributed to gramine's activity. It is important to note that, under the assay conditions, the higher solubility of gramine relatively to the other alkaloids tested, allowed us to find a strongest effect. We are not aware of the inhibition behavior of lupanine, sparteine and angustifoline in the same range of concentrations tested for gramine. Still, these compounds contribute to some extent for the extracts activity. The results obtained suggest that besides the phenolic compounds previously reported (Czubinski et al., 2012), alkaloids can play a role in LOX inhibition in lupin seeds.

•NO is a short-lived free radical that mediates many biological processes. One of its functions is to enhance the bactericidal and tumoricidal activities of activated macrophages. Excessive production of •NO could however potentially lead to tissue damage and activation of pro-inflammatory mediators (Adebayo et al., 2015).

The rich-alkaloid lupin extracts of WL-M-PT (1.383–0.086 mg of dried extract/mL), WL-M-IT (2.133–0.133 mg of dried extract/mL), WL-E (0.917–0.057 mg of dried extract/mL), YL-N (3.867–0.242 mg of dried extract/mL), YL-D (0.617–0.039 mg of dried extract/mL), YL-T (0.533–0.033 mg of dried extract/mL), NLL-A (2.217–0.139 mg of dried extract/mL) and NLL-S (0.733–0.046 mg of dried extract/mL) were tested. Solubility issues precluded the evaluation of higher concentrations. All the extracts displayed weak activity against •NO, NLL-A displaying the best scavenging activity (20% at the highest concentration; data not shown).

Pure compounds confirmed the low solubility revealed by the extracts. Gramine was able to be tested at higher concentrations than the other alkaloids, scavenging •NO up to 34% at the maximum concentration (1 mg gramine/mL; data not shown). Lupanine (0.238 mg/mL) presented ca. 11% of activity, whereas sparteine and angustifoline revealed no activity (data not shown).

The extracts and the pure alkaloids themselves did not prove to be promising scavengers of •NO. These results seem to be in accordance with previous works, as lupins present similar or lower antioxidant potential than other grain legumes (Khan et al., 2015), the relation between antioxidant activity and chemical composition being not clear. Some works relate lupins antioxidant activity with the levels of phenolic compounds (Siger et al., 2012), others state that there is no correlation between total phenolics and the observed activity, as carotenoids, tocopherols, peptides and polysaccharides from lupins seeds also appear to give a contribution (Khan et al., 2015; Thambiraj et al., 2015). To our knowledge, there is no information yet about lupin alkaloids role. The results obtained indicate that they are not scavengers of •NO.

It should be noted, however, that rich-alkaloid extracts were tested as a whole. Therefore, the activity observed may also result from synergism and/or antagonism phenomena that occur between the several constituents, which are difficult to predict. In addition, regarding LOX activity, allosteric effects must be taken into account, since the various LOX isozymes can act on multiple substrates, producing a variety of products with unique biological effects (Jameson et al., 2015). In addition, it should be highlighted that results obtained in *in vitro* cell-free systems may not be confirmed with cellular assays. Additionally, biological effects observed *in vivo* or in cellular assays may be enhanced, depending, for example, on metabolic processes occurring in the body. In this sense, this work represents a first approach.

4. Conclusion

The results obtained allowed to establish for the first time the alkaloids profile of some lupin samples (Lumen, Estoril, Nacional and Azuro). The need of monitoring the alkaloids composition of lupins is here highlighted, since values obtained for Multitalia, a known Italian variety, are quite higher than those usually observed. The production of Polish YL in Mediterranean conditions appears to result in lower alkaloid levels in the seed; nevertheless, other varieties should be investigated in this regard. The studied rich-alkaloid lupin extracts showed moderate LOX inhibitory activity, explained, at least partially, by their alkaloid composition, but were weak •NO scavengers.

The results improve the knowledge and encourage the use of this crop, not only as whole seed and flour, as it is traditionally consumed, but as nutraceuticals or therapeutic agents.

As the alkaloids were extracted with an aqueous solvent, the method used herein may be of particular interest from economic and environmental points of view. It could be applied on a large-scale context using a closed circuit, with which the toxicity of more aggressive solvents, e.g. dichloromethane, is avoided. As the resulting extract is a dried extract, its safety is ensured. Anyway, since the extraction yields obtained are relatively low, other extractive processes, preferably clean green procedures, such as super critical fluid extraction (Nossack et al., 2000), must be considered.

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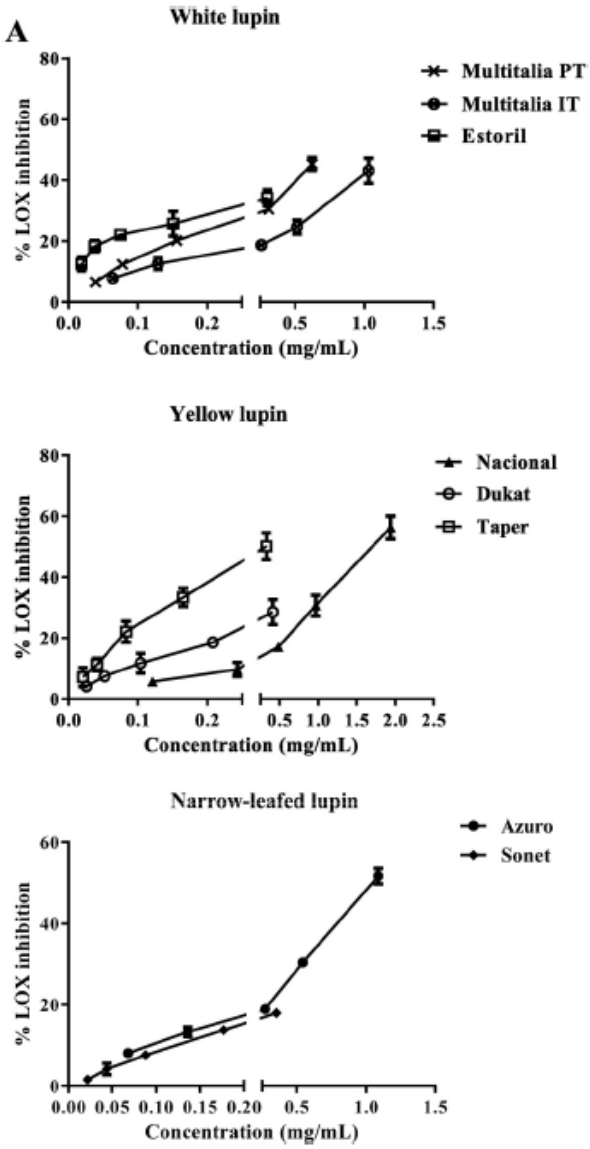

Fig. 3. 5-LOX inhibitory effect of white, yellow and narrow-leafed lupins' rich-alkaloid extracts (A) and of pure alkaloid standards (B).

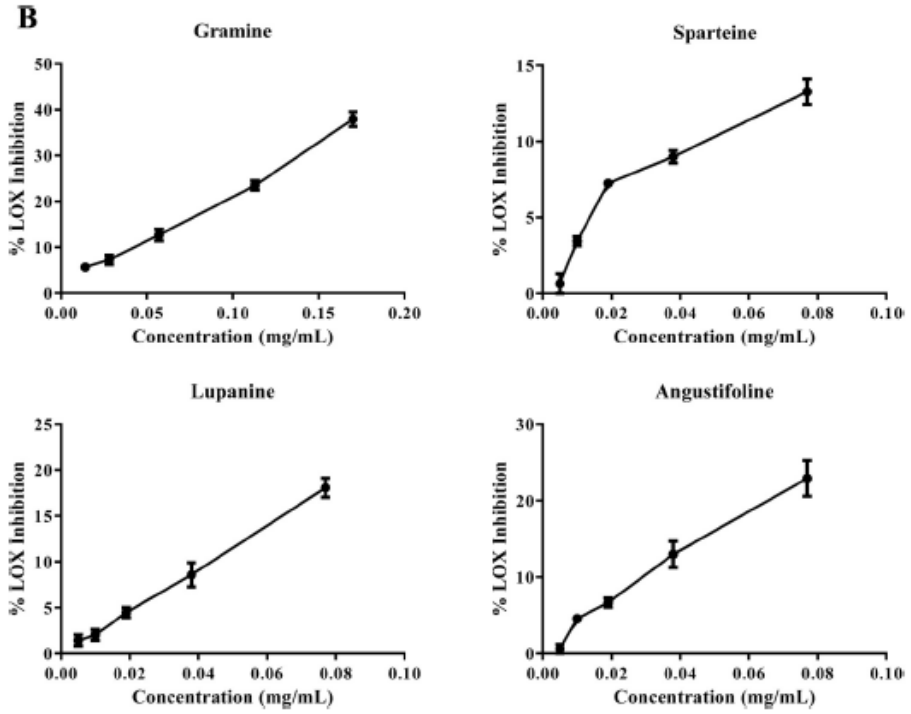

Fig. 3. (*Continued*)

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CHAPTER 7: APPARENT DIGESTIBILITY COEFFICIENTS OF EUROPEAN GRAIN LEGUMES IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AND NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

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Apparent digestibility coefficients of European grain legumes in rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*)

S.C.Q. Magalhães[1] | A.R.J. Cabrita[1] | P. Valentão[2] | P.B. Andrade[2] | P. Rema[3,4] | M.R.G. Maia[1,5] | L.M.P. Valente[6,7] | A.J.M. Fonseca[1]

[1]REQUIMTE, LAQV, ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Porto, Portugal

[2]REQUIMTE, LAQV, Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

[3]Universidade de Trás-os-Montes e Alto Douro (UTAD), Vila Real, Portugal

[4]CECAV-Universidade de Trás-os-Montes e Alto Douro (UTAD), Vila Real, Portugal

[5]REQUIMTE, LAQV, DGAOT, Faculdade de Ciências, Universidade do Porto, Porto, Portugal

[6]ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Porto, Portugal

[7]CIIMAR-CIMAR L.A., Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, Porto, Portugal

Correspondence
A.J.M. Fonseca, REQUIMTE, LAQV, ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Porto, Portugal.
Email: ajfonseca@icbas.up.pt

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Abstract

Two trials were carried out to evaluate the apparent digestibility coefficients (ADCs) of dry matter (DM), crude protein (CP), lipids, starch and gross energy (GE) of six European varieties of grain legumes, namely chickpea-type Kabuli (CHK), chickpea-type Desi (CHD), field pea (FP), faba bean (FB), white lupin (WL) and chickling vetch (CV), in rainbow trout and Nile tilapia juveniles. The ADCs were measured using a reference diet and six experimental diets (700 g/kg of the reference diet and 300 g/kg of each raw grain legume) containing 10 g/kg chromic oxide as inert marker. Additionally, grain legumes were analysed for the organic acids profile. In rainbow trout, FP presented the highest ADCs of DM, CP and GE, whereas chickpeas and FB had the lowest DM, GE and starch ADC values. In Nile tilapia, the lowest values of nutrients (except starch) and energy ADCs were found for FP and CV. Nutrients and energy of chickpeas, WL and FB were better digested by tilapia, whereas FP was better digested by trout. Overall results reveal raw grain legumes as promising feed sources for both fish species.

KEYWORDS
digestibility, grain legumes, Nile tilapia, organic acids, rainbow trout, raw seeds

1 | INTRODUCTION

The quest for alternative ingredients as replacement of fish meal (FM) in aquafeeds has been driven by FM declining supply and simultaneous expansion of the aquaculture sector worldwide. Plant protein sources, and particularly soybean meal (SBM), have been largely used to replace FM in feed formulations for farmed fish species (Catacutan, Coloso, & Acosta, 2015). However, due to markets unpredictability and feed

costs fluctuations, related to the strong dependency on imported FM and SBM, it urges to explore the feasibility of using locally produced ingredients in Europe.

Grain legumes, with a protein content roughly ranging between 220 and 380 g/kg, constitute low-price ingredients (Fagbenro, 1998) and offer a certain flexibility to the feed manufacturer as they might replace both protein and energy dietary sources (Cruz-Suarez, Ricque-Marie, Tapia-Salazar, McCallum, & Hickling, 2001). Besides its overall high nutritional value, legume seeds also contain several non-nutritive compounds that result from plant's secondary metabolism, capable of exerting positive, negative or both effects when ingested on a regular basis (Champ, 2002). The effects of many vegetable antinutritional factors (e.g., trypsin inhibitors, tannins, phytic acid and alkaloids) on fish digestibility, growth and performance were reviewed by Francis, Makkar, and Becker (2001). Several other metabolites exist that are able to promote health benefits on fish, but little information about their effects on fish species is available. Organic acids are examples of such metabolites with benefits related to the reduction in gut pH in fish, and consequent increased disease control, nutrient digestibility, minerals availability, phytase efficacy and, therefore, growth performance (Ng, Koh, Sudesh, & Siti-Zahrah, 2009; Shah, Afzal, Khan, Hussain, & Habib, 2015).

The apparent digestibility coefficients (ADCs) of grain legumes, including chickpeas, lupins, field peas, faba beans and vetches, were determined on various fish species including rainbow trout (Glencross & Hawkins, 2004; Gomes, Rema, & Kaushik, 1995; Thiessen, Campbell, & Adelizi, 2003; Tiril, Karayucel, Alagil, Dernekbasi, & Yagci, 2009), Atlantic salmon (Glencross et al., 2004), turbot (Burel, Boujard, Tulli, & Kaushik, 2000), Australian silver perch (Allan et al., 2000) and Nile tilapia (Fagbenro, 1998; Fontainhas-Fernandes, Gomes, Reis-Henriques, & Coimbra, 1999). In these studies, while crude protein (CP) digestibility coefficients values are generally above 0.80, gross energy (GE) ADC values show great variation in raw seeds, in most cases varying between 0.50 and 0.70. As the nutritive and non-nutritive values of grain legumes (e.g., peas and lentils) were shown to be strongly dependent on the legume variety (Wang & Daun, 2004, 2006; Wang, Hatcher, & Gawalko, 2008), the present study aimed at determining, for the first time, the digestibility of nutrients and energy of commercially available varieties of Portuguese grain legumes seeds in two important aquaculture species, rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*), with distinct feeding habits (carnivorous and omnivorous, respectively).

2 | MATERIAL AND METHODS

2.1 | Ingredients origin and experimental diets

The selected grain legumes are Portuguese varieties, registered in the Portuguese catalogue of varieties (CNV, 2015), and are part of the European plant variety database (PVD, 2015), being easily traded within the European Union and worldwide. All grain legumes were provided by Instituto Nacional de Investigação Agrária e Veterinária (INIAV, I.P., Elvas, Portugal) and included chickpea (*Cicer arietinum* L.)-type Kabuli (CHK; large, white to cream seeds) var. Elixir, and chickpea-type Desi (CHD; small and dark seeds) var. Elmo, field pea var. Pixel (FP; *Pisum sativum* L.), faba bean var. Favel (FB; *Vicia faba* L. minor), sweet white lupin var. Estoril (WL; *Lupinus albus* L.) and chickling vetch var. Grão-da-gramicha (CV; *Lathyrus cicera* L.). Seeds were dried in a forced-air oven (65°C, 24 hr) and grounded to pass through a 1-mm screen (0.5 mm for starch) before chemical analysis, and further grounded to pass through a 0.5-mm screen for fish diets manufacturing.

Based on known nutritional requirements of rainbow trout and Nile tilapia, a commercial-based reference diet (REF) was formulated for each species (Table 1). Both REF diets were produced by SPAROS Lda (Olhão, Portugal) using a pilot-scale twin-screw extruder. Oil was added after the extrusion process. For each fish species, seven diets were tested, the REF diet and six test diets each comprising 700 g/kg of the REF and 300 g/kg of one selected grain legume (particle size of 0.5 mm). Chromic oxide (Cr_2O_3; 10 g/kg) was added to all diets (REF and test diets), which were subsequently pelleted dry without steam, using a laboratory pellet press (CPM, C-300 model, San Francisco, USA) with a 4-mm die, and stored at 4°C until use.

2.2 | Fish and rearing conditions

Experiments were conducted by trained scientists (following the recommendations of the Federation of European Laboratory Animal

TABLE 1 Ingredient composition of the reference diets for rainbow trout and Nile tilapia (g/kg)

	Basal-mix trout	Basal-mix tilapia
Fish meal	320	70.0
Corn gluten	60.0	100
Wheat gluten	60.0	–
Rice protein concentrate	–	50.0
Soybean meal 48	150	240
Rapeseed meal	120	100
Wheat meal	120	90.0
Wheat bran	–	170
Corn meal	–	50.0
Fish oil	55.0	–
Soybean oil	90.0	49.0
Vitamin and mineral premix[a]	10.0	10.0
Binder	3.00	10.0
Antioxidant	2.00	2.00
Dicalcium phosphate	–	26.0
L-Lysine	–	10.0
L-Threonine	–	3.00
DL-Methionine	–	20.0

[a]Covered known requirements for rainbow trout and Nile tilapia (supplied by SPAROS Lda., Olhão, Portugal).

Science Associations—FELASA) according to the European Economic Community Animal Experimentation Guidelines Directive of 22 September 2010 (2010/63/EU).

2.2.1 | Apparent digestibility measurements in rainbow trout

The ADCs of the dietary components were measured by the indirect method using a diet replacement method as described by Cho and Slinger (1979). Faeces were removed from water by continuous filtration using the Choubert System (Choubert, Delanoue, & Luquet, 1982). Approximately 20 s passed since the collection of the faeces by one of the system's nets until they were deposited in a tray.

Seven homogenous groups of 12 rainbow trout juveniles (average weight of 80 ± 1.2 g), raised in UTAD Experimental Research Station (Vila Real, Portugal), were allotted to seven cylindrical tanks (water column: 100 cm; volume: 120 L; water flow rate: 7.5 L/min, average density: 8.0 g/L) supplied with filtered freshwater. Fish were acclimated to the new tanks during a two-week period during which they were fed the REF diet, once a day (10:00 a.m.). After acclimation, the diets were randomly assigned to the tanks. The first 5 days were used for acclimation to the new feed and no faeces were collected, followed by a seven-day experimental period for faeces collection. The first 5 days for acclimation at the start of each 12-day period was deemed sufficient for the fish to achieve complete evacuation of previous meals and therefore prevent faeces mixture. This procedure was repeated for each diet to replicate the results (two different periods; $n = 2$). To reduce the tank effect, diets in the second period were allocated to different tanks from those of the first period. Fish were fed once daily at 10.00 a.m. until visually satiated and feed was carefully distributed to fish avoiding any feed waste. Before feeding, faeces were collected from trays, pooled by tank and frozen at −20°C. At the end of the experimental period, faeces collected from each tank were freeze-dried, prior to analysis. Before changing diets, the fish were fasted for 24 hr to help ejecting the previous diet.

During the trial, the temperature ranged from 15.0 to 15.5°C and pH from 7.1 to 7.2. Water quality was monitored regularly and ammonia and nitrite concentrations were kept at optimal levels described for the species.

2.2.2 | Apparent digestibility measurements in Nile tilapia

The same faeces collection procedure was used for digestibility measurements in Nile tilapia, as successfully reported by Pereira, Valente, Sousa-Pinto, and Rema (2012). This system, typically used for salmonid studies, was adapted to a tank system supplied with thermoregulated and recirculated freshwater. Seven homogenous groups of 20 Nile tilapia (average weight of 55 ± 1.0 g), previously raised in UTAD Experimental Research Station (Vila Real, Portugal), were allocated to seven cylindrical tanks (water column: 100 cm; volume: 120 L; water flow rate: 5.2 L/min; average density: 9.2 g/L), similar to the described for rainbow trout experimental design. During the trial, the temperature ranged from 25 to 26°C and pH from 6.9 to 7.1. Water quality was monitored regularly and ammonia and nitrite concentrations were kept at optimal levels described for the species. The feeding protocol was similar to that used with rainbow trout.

2.2.3 | Calculations

The ADC of nutrients and GE for the REF and experimental diets were calculated according to Maynard, Loosli, Hintz, and Warner (1979) as follows:

$$\text{ADC} = 1 - (\text{dietary } Cr_2O_3/\text{faeces } Cr_2O_3) \times (\text{faeces nutrients or GE level/dietary nutrient or GE level})$$

ADC of dry matter (DM) was calculated according to the following:

$$\text{ADC} = 1 - \text{dietary } Cr_2O_3/\text{faeces } Cr_2O_3$$

The ADC of the test ingredients was estimated as proposed by Forster (1999):

$$\text{ADC} = \text{ADC}_{\text{test diet}} + [(\text{ADC}_{\text{test diet}} - \text{ADC}_{\text{ref diet}}) \times ((0.7\times \text{nutrients or GE of ref diet}) / (0.3\times \text{nutrients or GE level of test ingredient}))]$$

2.3 | Analytical methods

2.3.1 | Proximate composition of test ingredients, diets and faeces

Test ingredients, experimental diets and freeze-dried faeces were finely milled and homogenized prior to analysis. According to AOAC (2000), DM of all samples was calculated after drying at 103 ± 2°C for 2 hr (method 930.15); CP content was determined using a Leco nitrogen analyser (method 990.03) and calculated as $N \times 6.25$ (method 954.01). Total lipid content of all samples was determined according to Folch, Lees, and Sloane Stanley (1957) and starch according to Thivend, Mercier, and Guilbot (1972). Neutral detergent fibre (assayed with heat-stable amylase and expressed exclusive of residual ash; aNDFom), acid detergent fibre (ADF; expressed inclusive of residual ash) and lignin (determined by solubilization of cellulose with sulphuric acid and expressed exclusive of residual ash) of the ingredients were determined by the procedures of Van Soest, Robertson, and Lewis (1991) and Robertson and Van Soest (1981). Gross energy of all samples was determined in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany). Chromic oxide content of diets and faeces was determined according to Bolin, King, and Klosterman (1952); samples were digested at 220°C in a Kjeldahltherm block digestion unit, and absorbance was determined at 440 nm. All analyses were run in duplicate. Non-starch carbohydrates of the test ingredients were calculated by deducting the sum of ash, CP, total lipids and starch from DM. Digestible energy of the REF and experimental diets was calculated as ADC of GE × GE.

2.3.2 | Organic acids composition of test ingredients

Organic acids from grain legumes were extracted as described by Taveira et al. (2009) with slight modifications. Briefly, each sample was extracted twice with methanol/water (1:1). The combined supernatants were evaporated to dryness under reduced pressure, at 30°C, redissolved in water acidified to pH 2 and applied in the C18 column (non-end-capped; 50 μm particle size, 60 Å porosity; 10 g of sorbent mass/70 ml of reservoir volume; Chromabond, Macherey-Nagel, Germany), previously conditioned with 70 ml of methanol and 30 ml of acidified water. The aqueous fraction collected was dried under vacuum, redissolved in an appropriate volume of sulphuric acid 0.01 N, and membrane-filtered (0.45 μm). The organic acid-rich extracts were then analysed according to Sousa et al. (2007), using an analytical HPLC-UV unit (Gilson, Villiers-le-bel, France) with an ion exclusion column, Nucleogel® Ion 300 OA (300 × 7.7 mm; Macherey-Nagel, Germany). Elution was carried out in isocratic mode with sulphuric acid 0.01 N, under a flow rate of 0.2 ml/min and volume of injection of 20 μl. Detection was achieved at 214 nm. Quantification of organic acids of grain legumes extracts was based on the absorbance recorded in the chromatograms relative to external standards, namely oxalic, pyruvic, malic, fumaric, aconitic and citric acids (Sigma, St. Louis, MO). The data were processed on a Clarity Software system (Prague, Czech Republic). Each extract was injected in triplicate.

2.4 | Statistical analysis

All statistical analyses involving the experimental data were performed using SPSS® v.22 (IBM, USA). For each fish species, mean values of ADCs of test diets and ingredients were compared by one-way analysis of variance (one-way ANOVA) with DM, CP, lipids, starch and GE as dependent variables. Tukey's HSD *post hoc* test was used to compare means. In all cases, significant differences were considered when $p < .05$.

3 | RESULTS

3.1 | Chemical composition

The proximate composition and organic acid contents of the ingredients (expressed as DM basis) are shown in Table 2. Crude protein content ranged between 223 and 250 g/kg among all grain legumes except WL that had the highest CP content (363 g/kg). Lipids were lowest for CV (23.8 g/kg) and highest for WL (107 g/kg). The aNDFom, ADF and lignin contents varied among grain legumes. The WL presented the highest aNDFom content (231 g/kg) and CHK the lowest ADF content (30.0 g/kg). Starch ranged from 389 g/kg in CV to 432 g/kg in FP, whereas in WL it was not detected. Oxalic, aconitic, citric, pyruvic, malic and fumaric acids were the organic acids identified in grain legumes, with total contents ranging between 1.24 and

TABLE 2 Proximate and organic acids composition (g/kg) of the test ingredients (dry matter basis)[a]

	CHK	CHD	FP	FB	WL	CV
Proximate composition						
Ash	28.3	33.7	32.8	40.2	37.7	36.6
Crude protein	237.2	220.2	236.4	249.5	362.8	222.6
Lipids	62.5	41.6	34.5	26.6	107.0	23.8
aNDFom[b]	142.8	228.5	197.1	204.1	230.6	226.0
Acid detergent fibre[c]	30.0	94.3	66.3	102.6	155.9	92.4
Lignin[b]	16.9	15.1	6.3	20.5	8.2	13.5
Starch	412.5	344.5	432.4	409.0	n.d.	388.5
Non-starch carbohydrates	262.9	370.0	271.3	256.7	450.7	331.2
Gross energy (MJ/kg)	17.5	16.4	15.8	15.9	18.3	16.1
Organic acids						
Oxalic acid	0.07	0.06	n.d.	n.d.	0.05	0.04
Aconitic acid	0.01	0.00	0.00	0.01	0.00	0.00
Citric acid	2.56	1.10	0.98	0.97	3.62	1.07
Pyruvic acid	n.d.	n.d.	n.d.	0.23	n.d.	0.02
Malic acid	0.31	0.22	0.26	0.57	0.13	0.21
Fumaric acid	0.00	0.00	0.00	n.d.	0.00	0.00
Total	2.95	1.39	1.24	1.78	3.80	1.34

[a]Values are expressed as mean ($n = 2$).
[b]Expressed exclusive of residual ash.
[c]Expressed inclusive of residual ash.
CHK, chickpea-type Kabuli; CHD, chickpea-type Desi; FP, field pea; FB, faba bean; WL, white lupin; CV, chickling vetch; aNDFom, neutral detergent fibre assayed with heat-stable amylase; n.d., not detected.

3.80 g/kg in FP and WL, respectively. Citric acid was the major compound found (0.97–3.62 g/kg) in all grain legumes.

Proximate composition of the experimental diets is shown in Table 3. Reference diets from both rainbow trout and Nile tilapia showed higher CP content than the test diets. Among test diets fed to rainbow trout, CHK had the highest levels of lipids and starch (161 and 157 g/kg, respectively). The REF diet fed to Nile tilapia had the greatest lipid levels (106 g/kg), whereas the FP diet had the lowest level (54.2 g/kg). Starch content was highest in the FP diet (224 g/kg).

3.2 | Apparent digestibility coefficients

Digestibility coefficients are presented in Table 4 for rainbow trout and in Table 5 for Nile tilapia. In rainbow trout, CHK, CHD and FB diets had a significantly lower ($p < .001$) ADC of DM (0.67–0.69) than the FP, WL and CV diets (0.74–0.77). Crude protein and lipid ADCs of rainbow trout diets were always above 0.90. Chickpea diets had lower ($p < .01$) starch and GE ADCs (0.06–0.08 and 0.72, respectively) than FP and CV diets (0.33–0.36 and 0.78–0.82, respectively). Regarding ingredients, FP had the highest ADCs for DM (0.87), CP (1.00) and GE

TABLE 3 Proximate composition and energy content (g/kg or MJ/kg dry matter basis) of the reference and experimental diets[a]

	REF	CHK	CHD	FP	FB	WL	CV
Rainbow trout							
Dry matter	945.8	933.3	933.3	933.8	933.9	942.2	933.0
Ash	158.6	121.5	119.7	126.4	126.5	120.3	123.3
Crude protein	417.2	369.0	361.5	379.0	382.5	389.0	372.3
Lipids	156.4	160.9	118.9	88.4	106.5	121.9	114.7
Starch	74.2	157.0	135.1	136.2	134.9	42.8	127.2
Gross energy	18.9	18.2	18.0	17.9	17.8	18.8	17.8
Nile tilapia							
Dry matter	911.1	913.0	909.3	914.4	912.9	921.0	912.0
Ash	93.0	75.9	79.3	79.8	81.7	79.5	80.7
Crude protein	382.0	331.8	331.3	336.8	333.7	361.9	336.1
Lipids	105.9	64.4	74.8	54.2	89.6	85.6	92.4
Starch	132.3	203.6	202.5	224.4	212.9	81.5	197.6
Gross energy	16.5	16.9	16.7	16.6	17.2	17.4	16.4

[a]Values are expressed as mean ($n = 2$).

REF, reference diet; CHK, chickpea-type Kabuli; CHD, chickpea-type Desi; FP, field pea; FB, faba bean; WL, white lupin; CV, chickling vetch.

TABLE 4 Apparent digestibility coefficients (ADC) of nutrients and energy of experimental diets and feed ingredients for rainbow trout

	REF	CHK	CHD	FP	FB	WL	CV	*SEM*	*p*
ADC diet									
Dry matter	0.732	0.673^{b}	0.688^{b}	0.773^{a}	0.673^{b}	0.744^{a}	0.758^{a}	0.011	***
Crude protein	0.906	0.911^{b}	0.906^{b}	0.933^{a}	0.902^{b}	$0.921^{a,b}$	$0.917^{a,b}$	0.005	**
Lipids	0.938	0.957^{a}	0.952^{a}	0.930^{b}	0.919^{b}	0.949^{a}	0.952^{a}	0.003	***
Starch	0.380	0.063^{b}	0.081^{b}	0.329^{a}	0.141^{b}	0.155^{b}	0.363^{a}	0.038	**
Gross energy	0.793	0.718^{c}	0.718^{c}	0.819^{a}	$0.730^{b,c}$	0.788^{a}	$0.778^{a,b}$	0.014	**
Digestible energy (MJ/kg dry matter basis)	14.9	13.0^{b}	12.9^{b}	14.6^{a}	13.0^{b}	14.8^{a}	$13.9^{a,b}$	0.003	**
ADC ingredient									
Dry matter		0.540^{b}	0.583^{b}	0.868^{a}	0.538^{b}	0.770^{a}	0.818^{a}	0.036	***
Crude protein		$0.931^{a,b}$	$0.909^{a,b}$	1.000^{a}	0.890^{b}	$0.962^{a,b}$	$0.969^{a,b}$	0.026	*
Lipids		1.000^{a}	1.000^{a}	0.851^{b}	0.656^{c}	0.989^{a}	1.000^{a}	0.032	***
Starch		0.000^{b}	0.000^{b}	0.309^{a}	0.040^{b}	n.d.	0.355^{a}	0.044	***
Gross energy		$0.531^{b,c}$	0.517^{c}	0.892^{a}	$0.557^{b,c}$	0.777^{a}	$0.738^{a,b}$	0.052	**

Means in the same row with different superscript letters are significantly different: *$p < .05$; **$p < .01$; ***$p < .001$.

SEM, standard error of the mean ($n = 2$); REF, reference diet; CHK, chickpea-type Kabuli; CHD, chickpea-type Desi; FP, field pea; FB, faba bean; WL, white lupin; CV, chickling vetch; n.d., not determined.

TABLE 5 Apparent digestibility coefficients (ADC) of nutrients and energy of experimental diets and feed ingredients for Nile tilapia

	REF	CHK	CHD	FP	FB	WL	CV	SEM	*p*
ADC diet									
Dry matter	0.708	0.780^{a}	0.770^{a}	0.725^{b}	0.775^{a}	0.757^{a,b}	0.728^{b}	0.010	**
Crude protein	0.857	0.882^{a}	0.882^{a}	0.879^{a}	0.891^{a}	0.895^{a}	0.845^{b}	0.007	**
Lipids	0.904	0.895	0.925	0.850	0.919	0.932	0.919	0.023	n.s.
Starch	1.000	0.941	0.960	0.968	0.958	0.999	0.963	0.035	n.s.
Gross energy	0.692	0.777^{a}	0.753^{a,b}	0.706^{b}	0.764^{a}	0.760^{a}	0.702^{b}	0.014	**
Digestible energy (MJ/kg dry matter basis)	11.4	13.1^{a}	12.5^{a,b}	11.7^{b,c}	13.2^{a}	13.2^{a}	11.5^{c}	0.002	**
ADC ingredient									
Dry matter		0.938^{a}	0.909^{a}	0.764^{b}	0.922^{a}	0.866^{a,b}	0.773^{b}	0.031	**
Crude protein		0.973^{a}	0.984^{a}	0.962^{a}	1.000^{a}	0.988^{a}	0.796^{b}	0.021	**
Lipids		0.861^{a}	1.000^{a}	0.463^{b}	1.000^{a}	0.996^{a}	1.000^{a}	0.091	**
Starch		0.897	0.924	0.945	0.926	n.d.	0.933	0.069	n.s.
Gross energy		0.965^{a}	0.895^{a,b}	0.740^{b}	0.938^{a}	0.903^{a,b}	0.725^{b}	0.045	**

Means in the same row with different superscript letters are significantly different: **$p < .01$; ***n.s., non-significant ($p > .05$).
SEM, standard error of the mean ($n = 2$); REF, reference diet; CHK, chickpea-type Kabuli; CHD, chickpea-type Desi; FP, field pea; FB, faba bean; WL, white lupin; CV, chickling vetch; n.d., not determined.

(0.89), although similar to those of WL and CV (0.77–0.82, 0.96–0.97 and 0.74–0.78, respectively). Starch ADC was null in both chickpeas and extremely low in FB (0.04), while FP and CV presented the highest ($p > .001$) values (0.31–0.36). No significant differences ($p > .05$) were observed for nutrients and GE ADC values between both chickpeas and FB, except for lipids.

In Nile tilapia, dietary ADCs of DM were lower ($p < .01$) in the FP and CV diets (0.73) than in the other diets (0.76–0.78). The ADC of CP was lowest ($p < .01$) in the CV diet (0.85), with no differences between the other diets (0.88–0.90). No significant differences ($p > .05$) were observed between diets for lipid and starch ADCs ($p > .05$). The ADC of GE was highest ($p < .01$) in CHK, FB and WL diets (0.76–0.78). Regarding ingredients, CV presented the lowest CP ADC (0.80), differing significantly ($p < .01$) from all other varieties. Lipid ADC was lowest ($p < .01$) for FP (0.46 versus 0.86–1.00). Starch ADC did not vary significantly among grain legumes ($p > .05$), but GE ADC was lowest ($p < .01$) in CV and FP (0.73–0.74) and highest in CHK and FB (0.94–0.97).

4 | DISCUSSION

The selected grain legume varieties showed quite similar nutrient and GE contents (Table 2), with the exception of WL that had a higher CP, lipids, aNDFom, ADF and non-starch carbohydrate contents than all the other varieties. Chickpea-type Kabuli had higher levels of lipids and starch and lower levels of fibre compared to CHD, as previously reported by Bampidis and Christodoulou (2011). Conversely, CV had a lower CP content than that reported by Hanbury, White, Mullan, and Siddique (2000; 296 g/kg). Starch levels of FP, FB and CV were below the values reported in other varieties, whereas the opposite was observed for aNDFom contents (Bampidis & Christodoulou, 2011; Hanbury et al., 2000; Jezierny, Mosenthin, & Bauer, 2010). The lack of detection of starch in WL agrees with the reported low or no levels of this nutrient in *Lupinus* spp. seeds (Jezierny et al., 2010; Petterson, 2000). Crude protein content of grain legumes was lower than that previously reported for SBM (480 g/kg; Drew, Borgeson, & Thiessen, 2007).

Among organic acids, citric acid was the major compound found in all grain legumes evaluated in the present study, with values ranging from 1 to 4 g/kg. This organic acid has been extensively used in aquafeeds for acidification. Previous studies showed that the supplementation of diets for rainbow trout, red seabram (*Pagrus major*) and rohu (*Labeo rohita*) with 10–30 g/kg citric acid induced positive results in terms of growth and feed utilization (Shah et al., 2015). All grain legume varieties evaluated in this study could hence be considered a natural source of this organic acid to be included in aquafeeds. Although oxalic acid seems to affect protein digestion and calcium and magnesium metabolism in monogastrics (Francis et al., 2001), it was absent in both FP and FB, whereas the other studied varieties had a content even lower than that reported for soybean seeds (Massey, Palmer, & Horner, 2001).

The digestibility of Portuguese legume grains was not previously evaluated, so selected grains were tested in two important farmed species. The ADC approach herein used followed the classic methodology and ingredients were combined with basal diets in a 70:30 ratio (NRC, 2011).

The present results showed that rainbow trout was very efficient in using dietary lipids and protein of grain legumes, with ADC values in experimental and REF diets always above 0.90. Crude protein ADCs of the ingredients themselves were also high (>0.89). Studies by Gomes et al. (1995) have also examined the nutritional value of whole raw grain legumes, such as pea and faba bean (unknown varieties), when fed to rainbow trout, although they reported lower CP ADCs

than those observed in our study (0.80 versus 0.89–1.00). As the ADC and faecal collection methods used in both studies are identical, differences in digestibility values may be attributable to the varieties themselves. Direct comparisons of our study with those of Thiessen et al. (2003), Glencross and Hawkins (2004) and Tiril et al. (2009) with grain legumes in rainbow trout are difficult because of differences in faecal collection methods and also the processing state of the grain legumes used, namely extruded or kernel meals rather than the raw whole seeds used in the present study.

The dietary incorporation of both chickpeas and FB resulted in a pronounced negative effect on DM and GE digestibility coefficients when compared to the inclusion of the other ingredients. Grain legumes generally contain high levels of carbohydrates (starch and non-starch carbohydrates) and antinutritional factors (Francis et al., 2001) that can affect diet digestibility in most fish species, with a more pronounced impact on carnivorous fish. In the present study, the highly indigestible starch fraction of chickpeas (1.00) and FB (0.96) could partly explain the lowest ADCs of GE observed in rainbow trout. As far as we are aware of, reports on the use of raw chickpeas in carnivorous fish do not exist that on the inclusion of raw FB being consistent with our results (Gomes et al., 1995). In turn, extruded chickpeas and faba beans were reported to present overall nutrients and energy ADCs above 0.90 in rainbow trout (Tiril et al., 2009). In the present study, although CP and lipid fractions from chickpeas and FB were well digested, overall DM and GE ADCs of diets and ingredients could be improved by extrusion and autoclaving. These treatments when applied to chickpeas and faba beans have been reported to gelatinize starch, reduce endogenous antinutritional factors and improve the utilization of starch, fat and protein in pigs, poultry and rainbow trout (Bampidis & Christodoulou, 2011; Pfeffer, Kinzinger, & Rodehutscord, 1995). It is noteworthy that both chickpeas and FB herein studied also presented the highest levels of lignin (Table 2). Significant deleterious effects of lignin on CP, lipid and GE digestibility have been reported in rainbow trout (Glencross et al., 2008) and could also explain the significantly lower lipid ADC values observed in FB.

The highest overall ADC results in rainbow trout were obtained with FP, WL and CV. In a previous work, Thiessen et al. (2003) found that whole peas in extruded diets for rainbow trout resulted in low DM and GE ADCs due to their poorly digested starch fraction (0.14). In the present study, starch ADC of FP was 0.31, putatively contributing to the overall high ADCs obtained for this ingredient. Indeed, Wang et al. (2008) reported that variety and processing could affect the composition of field peas, with possible further impacts on their digestibility.

To the best of our knowledge, no published studies exist on the use of CV in aquafeeds. This study is therefore the first report on the use of seeds of *L. cicera* in rainbow trout and overall digestibility results appear promising for this grain legume. In contrast, lupins are widely considered in formulations for carnivorous fish (given their high levels of protein and lipids), including rainbow trout (Bórquez, Serrano, Dantagnan, Carrasco, & Hernandez, 2011; Burel et al., 2000). Alkaloids and fibre levels are the major constraints associated with lupin seeds. The WL used in the present work is a low-alkaloid variety (Magalhães et al., 2017), but almost half of it corresponds to non-starch carbohydrates (Table 2), a fraction generally poorly digested by fish, regardless of their feeding habits (Stone, 2003). Nevertheless, digestible energy of WL diet was high, and close to that of the REF diet, implying a high ADC value of the non-starch carbohydrate fraction.

The dietary inclusion of all grain legumes was well accepted by Nile tilapia as suggested by the GE ADCs of experimental diets. Nile tilapia is an omnivorous fish species mostly feeding on plant ingredients, thereby requiring less dietary protein and lipids and tolerating higher levels of carbohydrates for optimum performance in comparison with rainbow trout. Protein ADC was above 0.96 for all the studied grain legumes except CV (0.80). Also in Nile tilapia, Fontainhas-Fernandes et al. (1999) reported for raw whole seeds of WL, FP and FB protein ADCs ranging between 0.78 and 0.90, lower values than those presented herein. Differences may result from intervarietal variation or from the different methods used to collect the faeces (Guelph versus Choubert systems).

Likewise rainbow trout, as far as we are aware of, no published studies exist on the incorporation of CV in Nile tilapia or similar fish species. However, seeds of grass pea, another species of the *Lathyrus* genus (*Lathyrus sativus*), were evaluated in rohu, an omnivorous fish species of the carp family (Barse et al., 2004; Ramachandran, Bairagi, & Ray, 2005; Ramachandran & Ray, 2008). *Lathyrus* species contain high fibre levels and several antinutritional factors, namely protease inhibitors, tannins, phytates and lathyrogens (neurotoxic amino acid β-N-oxalyl-L-α,β-diaminopropionic acid or β-ODAP), which hinders free nutritional utilization (Hanbury et al., 2000). Ramachandran et al. (2005) verified that fermentation could reduce grass pea tannins, phytates, β-ODAP and crude fibre by 88%, 81%, 24% and 25%, respectively, further allowing fermented grass pea to be included up to 300 g/kg in rohu diets. Moreover, Ramachandran and Ray (2008) reported a significantly better growth performance and feed utilization of rohu fed 300 g/kg extruded, germinated and fermented grass pea compared with fish fed diets with raw seeds. These reports on another *Lathyrus* spp. seeds may indicate that processing is also needed for *L. cicera* grains when to be included in feed formulations for Nile tilapia. Field pea presented the poorest ADC results in terms of DM and GE (0.74–0.76) along with CV.

The best ADC values in Nile tilapia were obtained with chickpeas (CHK, CHD), FB and sweet WL. As far as we are aware of, no previous reports evaluated the potential of chickpeas in Nile tilapia. In silver perch (Booth, Allan, Frances, & Parkinson, 2001), whole and dehulled chickpeas presented lower digestibility coefficients compared with ours, when using the same ADC methodology but settlement as the faecal collection methods. The good ADC results herein obtained for raw chickpeas in Nile tilapia may indicate that these are interesting protein and energy sources for this fish species.

Faba beans, but especially lupins, are often included in diets for Nile tilapia. Azaza et al. (2009) have evaluated raw faba beans inclusion in isonitrogenous and isoenergetic diets in tilapia juveniles, using a distinct ADC determination approach to ours and stripping as the faeces collection method. They reported a decrease in overall dietary ADCs and growth performance of fish when faba beans inclusion in diets increased from 240 to 360 g/kg, which was attributed to the

low levels of methionine and the presence of tannins and phenolics in these seeds. In the present work, the ADCs of DM and GE of sweet WL approached 0.80, whereas other WL varieties previously tested presented around 0.70 (Allan et al., 2000; Fontainhas-Fernandes et al., 1999). It is worth noting that these later studies used the same ADC methodology of ours through different faeces collection methods (Guelph system and settlement, respectively). The herein obtained results appear to reinforce the idea that WL varieties are promising grains for Nile tilapia's diet in the unprocessed form.

5 | CONCLUSIONS

This work shows that raw grain legume varieties present potential in terms of digestibility to be included in diets for both rainbow trout and Nile tilapia. As processing only seems to be required for chickpeas and FB in rainbow trout and with CV in Nile tilapia diets, these grain legume varieties might be cost-effective alternatives to high-price fish meal and soybean meal. Chickpeas Kabuli and Desi showed similar digestibility coefficients, suggesting, at least at a digestibility level, that the inclusion of these seeds in diets for both fish species may be mainly dependent on their availability and price. However, CHK might have some advantage in relation to CHD due to its higher levels of citric acid. As this organic acid is usually supplemented in aquafeeds, the inclusion of CHK might be a more natural way of obtaining the required dietary acidification. The studied FP variety proved to be highly promising in diets for carnivorous fish despite its high starch content. Seeds of *L. cicera* were evaluated for the first time in diets for rainbow trout and Nile tilapia. The positive results herein obtained unravel the use of a new grain legume species in aquafeeds. Also, raw chickpeas were evaluated for the first time in both Nile tilapia and rainbow trout with very positive results in the former fish species.

To fully assess the real value of these grain legumes in aquaculture feed formulations, the next step would be to measure the ADCs of these grain legumes at different dietary inclusion levels, to perform growth and palatability trials, to study the immune status of fish and to determine the effects on the organoleptic quality of the fish.

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CHAPTER 8: GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

This dissertation contains four papers published in international journals and one manuscript to be further published. A detailed discussion and conclusions of each related experimental work are presented in previous chapters of this thesis, relatively to each study. A general discussion and conclusions of the studies carried out are presented in this chapter as well as some future perspectives.

8.1. General discussion

The European Union (EU) has been experiencing for several years now a serious deficit in protein sources of vegetable origin, one of the most affected sectors being the animal feed industry, since the massive production of meat/fish, milk and/or eggs requires a large contribution of compound feedstuffs in which vegetable protein is an essential component. The European Commission is now focused on reverting this situation by promoting and stimulating the local production of protein crops in the EU, with emphasis on grain legumes. The production of grain legumes is currently low in the EU mainly because of past economic and political decisions but there are several opportunities related to the cultivation and ingestion of these seeds by animals and humans that can contribute to promote them as crops as well as to disseminate their consumption (Chapter 1). However, there are also limitations that must be overcome to meet the goal of facing grain legumes as alternative vegetable protein sources in animal feeding, namely the need for more information on the nutritional value of these ingredients.

Portugal is an European country that also depends on large quantities of imported oilseed meals for compound feedstuffs. When reviewing in detail the particular situation of this country regarding grain legumes production during the last decades and use in animal feedstuffs (Chapter 2), it was noticed that there are several varieties of grain legumes well-adapted to national soil and climatic conditions, capable of growing under rainfed conditions (Autumn sowing), presenting final grain yields above those traditionally observed. Nonetheless, the information on the chemical composition of grain legumes used in Portugal, besides, mainly focused on crude protein, starch and ether extract.

To fill this gap on the lack of knowledge on the chemical composition of Portuguese and other European varieties, it urges, therefore, to characterize in depth several commercial grain legumes varieties. This was the aim of the present dissertation: to promote grain legumes incorporation in farmed animals' diets by unveiling their chemical composition. To achieve this goal, several varieties of different grain legumes species (a total of 51 samples), namely of chickpea (Kabuli and Desi

types), field pea, faba bean, lupins (white lupins, narrow-leafed lupins and yellow lupins), common vetch and chickling vetch were gathered from different European seed companies in their raw mature state to be assessed in terms of nutritive value and phytochemicals profile. If on the one hand, these grain legume species are adapted to the European edaphoclimatic conditions [1], on the other, they present most tradition in the European continent for food and/or feed purposes [2].

A detailed characterization of the primary metabolites, i.e. nutrients, of all varieties was performed and comprised proximate composition and fatty acids profile (Chapter 3). Indeed, the interest for an ingredient for food and feed arises primarily from its nutrients composition and content. The work presented on Chapter 3 can thus be a valuable tool for nutritionists, geneticists or producers dealing with animal feeding. It may allow creating a profile-type for grain legume species which could be of interest for the construction or improvement of nutritional tables. Nonetheless, other chemical parameters should be addressed in order to make grain legume profiles the most complete possible, namely, seeds amino acid profiles, minerals, vitamins and protein (albumins and globulins) and carbohydrate (mono-, di- and oligosaccharides) fractions.

Not only do nutrients matter when discussing vegetable ingredients. In fact, the knowledge on the compounds resulting from the plants' secondary metabolism (so-called non-nutrients) is essential to, on the one hand, avoid toxic episodes in animals and humans (in the case of antinutritional factors) and, on the other, take advantage of the beneficial effects that may arise thereof (e.g. biological activities such as antioxidant, anti-inflammatory activities, among others). Champ [3] stated that secondary metabolites may have positive, negative or both effects when ingested. This situation is simply illustrated for instance with phenolic compounds. They are widely known for their antioxidant power [4], however, phenolic compounds such as the condensed tannins can precipitate proteins from the diet, decreasing their bioavailability and increasing their fecal excretion, and also cause bitter taste to the diet, decreasing feed intake and subsequently impacting body weight gain [5]. For this reason, the profile of some phytochemicals in the seeds was also studied during the present thesis.

The choice regarding which phytochemicals to analyze fell on carotenoids (Chapter 3), organic acids (Chapter 3), phenolic compounds (Chapters 4 and 5) and alkaloids (in the specific case of lupins; Chapter 6). Carotenoids confer antioxidant properties to the seed and may also play a role on the color of the final product, this is, meat/fish or egg yolk [6, 7]. Dietary organic acids are commonly used as acidifiers in poultry and fish diets promoting growth and a better utilization of nutrients [8]. Phenolic

compounds contribute to the seed color and sensory characteristics of the seed and are responsible for several biological properties (e.g. antioxidant, anti-thrombotic, anti-inflammatory) [4]. Finally, alkaloids are major phytochemicals in lupin seeds causing, when at high concentrations in feedstuffs, bitter taste that affects diet palatability and body weight gain [9]. As a first approach in this regard, the characterization of grain legumes phytochemical profile was performed on a smaller group of varieties (n=30/51). All the varieties belonging to the Portuguese catalog of varieties [n=12; 10] were chosen for the analysis as well as others that, besides not Portuguese, were grown in the country (n=3). Additionally, depending on seed availability, other varieties were included (n=15). Adequate chromatographic techniques (HPLC-DAD, HPLC-UV, HPLC-DAD-ESI/MS^n, and GC-IT/MS) were used for this purpose.

The studies presented in chapters 3-6 greatly contributed to increase the knowledge on the secondary compounds of grain legumes with major advances being achieved. The individual phenolics profile of mature raw whole seeds of chickpea type Desi, field pea and common vetch was characterized for the first time whereas for chickpea type Kabuli, faba beans and lupins a further insight into their phenolics profile was achieved with the characterization of varieties/genotypes not studied to date (Chapter 4). The qualitative phenolics profile of the chickling vetch variety was in depth characterized constituting a great advance in terms of phytochemical knowledge of this seed and even of the *Lathyrus* genus as until then no seed of this genus had been analyzed in this regard. It was possible to detect in its composition the presence of glycosylated flavonoids mainly of the kaempferol type. It is therefore of major interest to determine also its quantitative profile of such compounds. Regarding lupins alkaloids, it was possible to establish for the first time the alkaloids of some lupin samples namely white lupin var. Lumen and Estoril, yellow lupin var. Nacional and narrow-leafed lupin var. Azuro (Chapter 6).

Applying a principal component analysis (PCA) on the sub-group of 30 samples for which nutrients and secondary compounds were studied (Table 1 of Chapter 3), it may be possible to understand the most prominent characteristics of each variety or group of varieties and thus better decide which one to choose depending, for example, on the animal species to fed (Figure 1). Indeed, the PCA, identify patterns that highlight similarities among samples. Noting, PCA did not consider the chickling vetch variety once its phenolic profile characterization was merely qualitative.

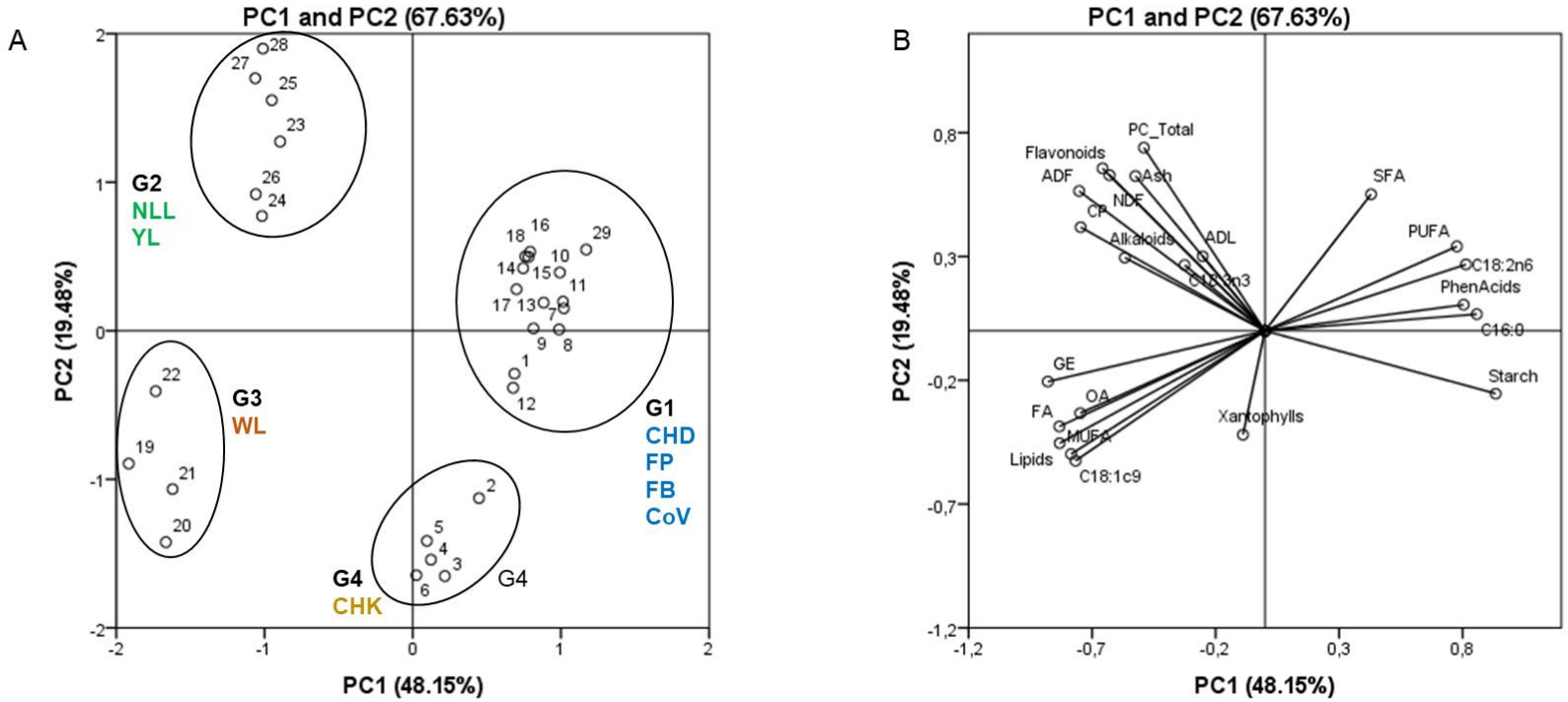

Figure 1. Projection of (A) grain legumes samples [variables: 1, chickpea type Desi (CHD) var. Elmo; 2, chickpea type Kabuli (CHK) var. Eldorado; 3, CHK var. Elixir; 4, CHK var. Elvar; 5, CHK var. Reale; 6, CHK var. Sultano; 7, field pea (FP) var. Esmeralda; 8, FP var. Grisel; 9, FP var. Marqueta; 10, FP var. Montrebei; 11, FP var. Montsant; 12, FP var. Pixel-I; 13, faba bean (FB) var Chiaro di Torrelama; 14, FB var. Diva; 15, FB var. Fabelle; 16, FB var. Favel; 17, FB var. Organdi; 18, FB var. Scuro di Torrelama; 19, white lupin (WL) var. Amiga; 20, WL var. Estoril; 21, WL var. Lumen; 22, WL var. Multitalia-PT; 23, narrow-leafed lupin (NLL) var. Azuro; 24, NLL var. Sonet; 25, yellow lupin (YL) var. Dukat; 26, YL var. Mister-PT; 27, YL var. Nacional; 28; YL var. Taper; 29, common vetch (CV) var. Barril] and (B) loadings by chemical and phytochemical contents into the plane composed by the principal components PC1 and PC2 containing 67.63% of total variance. (CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; GE, gross energy; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; OA, organic acids; PhenAcids., phenolic acids; PC_Total, total phenolic compounds).

Two PCs were retained and explained 67.63% of total data variability: PC1 represented 48.15%% of the variation and was highly associated with starch whereas PC2 represented 19.48% of data variability and was associated with total phenolic compounds content and in particular to that of flavonoids. Based on this, it is possible to notice how the different studied varieties are clearly grouped based on their composition in primary and secondary compounds, constituting four well-separated groups:

- Group G1 is composed by the type Desi chickpea, all field peas and faba bean varieties and common vetch. In comparison to the other varieties these grain legumes stand out for the highest levels in C16:0 and phenolic acids and the lowest contents in GE, lipids and FA; chickpea type Desi is the variety containing the greatest levels of xanthophylls, compounds that occur at very low concentrations in all the other grain legumes;
- Group G2 is composed by all narrow-leafed lupins and yellow lupin varieties. They present the highest levels of cell-wall components (NDF, ADF), flavonoids and total phenolic compounds; yellow lupin varieties are, in relation to all the other grain legumes, those with the greatest contents of ash and crude protein;
- Group G3 is composed by all white lupin varieties. These are richer than the others in terms of lipids, fatty acids, C18:1*c*9 and consequently monounsaturated fatty acids; additionally, white lupin varieties present also the highest levels of organic acids;
- Group G4 is composed by all Kabuli type chickpeas. These varieties present the highest levels of xanthophylls after chickpea type Desi but the poorest contents of overall phenolics, saturated fatty acids, C18:3*n*3 and cell-wall components.

This analysis, while encompassing all grain legume varieties, is comparing lupins with the other grain legume species and, as observed in Chapter 3, lupin species present marked differences to the remaining seeds species. In line with this, PCA clearly separated lupins from the other grain legumes by placing them in the negative plan of PC1. For grain legumes other than lupins, it is noticed that they all resemble except Kabuli chickpeas. The PCA is therefore useful to take a global vision of the chemical composition of all the grain legumes studied. It may suggest poor intra-specific deviations, at least for the chemical parameters herein analyzed, because varieties of a same species are all clustered together, however, as observed in Chapter 3 with the discriminant analysis based on crude protein content of the seeds, significant intraspecific differences may be observed for some nutrients. It is of interest in following studies to characterize carotenoids, organic acids, phenolic compounds and alkaloids also for the remaining 21 grain legume samples for which the profiles were not

determined and to see if these groups are maintained. Moreover, the secondary metabolites analyzed in the present dissertation in grain legume seeds should be faced as a starting point for the study of other phytochemical compounds. Enzyme inhibitors (protease and amylase inhibitors), lectins, saponins are some examples.

After an exhaustive study of several grain legume varieties, it matters to evaluate them *in vivo* to understand the impact of their inclusion in the diets of different farmed animals. The work developed in this sense in the present dissertation was based on two freshwater fish species of interest for the aquaculture industry, namely rainbow trout (carnivorous fish) and Nile tilapia (omnivorous fish). Since the first approach when an ingredient is to be tested in aquafeeds is the analysis of its apparent digestibility coefficients (ADC), this was herein developed (Chapter 7). Aquaculture is an expanding activity worldwide with very concrete needs regarding sustainable and cheap protein sources. In Chapter 2, it was evident the lack of *in vivo* nutrition studies on cataloged/commercial Portuguese varieties in the diet of farmed animals. Therefore, the evaluation of grain legumes apparent digestibility coefficients was built on six Portuguese varieties: chickpea type Desi var. Elmo, chickpea type Kabuli var. Elixir, field pea var. Pixel-I; faba bean var. Favel, white lupin var. Estoril and chickling vetch var. Grão-da-gramicha. Another breakthrough was achieved for chickling vetch as it was here evaluated for the first time in rainbow trout diets appearing as a promising ingredient for this fish species. Raw chickpeas were also studied for the first time in both species with very good results in Nile tilapia. Field pea was highly digestible in rainbow trout despite its high starch content. Processing appears to be needed for chickpeas and faba beans in rainbow trout and for chickling vetch in Nile tilapia diets to improve the overall digestibility of nutrients. Except for these situations, grain legume varieties could be cost-effective alternatives to high-price fish meal and soybean meal. Overall, Portuguese grain legumes digestibility results in farmed fish can boost the interest for Portuguese varieties, promoting their cultivation and commercialization. In fact, it is also possible to verify from Chapter 2 of this dissertation that Portuguese grain legume varieties present in some region of the country yields above 1000 kg/ha, which would be of interest to explore. To fully assess the real value of these grain legumes in aquaculture feed formulations, the next step would be to measure their ADCs at different dietary inclusion levels, to perform growth and palatability trials, to study the immune status of fish and to determine the effects on the organoleptic quality of the fish. Additionally, *in vivo* trials could be extended to other aquaculture species as well as to land farmed animals (poultry, swine and cattle) which end up being the major consumers of compound feedstuffs based on high-protein ingredients, as showed in Chapter 1.

The knowledge on European marketable varieties of grain legumes was, at different levels, improved along the works developed in the present thesis. Overall, all grain legumes

present potential as raw mature vegetable protein sources in diets for farmed animals. However, levels of alkaloids (in the case of lupins), starch or fibre shall be considered individually for each variety also taking into account the animal species to be fed.

Although it is not easy to totally replace an ingredient such as soybean meal in animal feed, it is crucial for Europe to have alternatives and to know about their potential as much as possible. The greater or lesser incorporation rates of grain legumes in European compound feedstuffs will depend on their price and availability. “Sustainability”, widely claimed nowadays for animal and vegetable production systems can in fact help increasing the area devoted to grain legumes in the EU and thus their inclusion in European compound feedstuffs.

8.2. Conclusions

At the beginning of the present thesis, we proposed to review the state of knowledge on grain legumes production in Portugal, to improve the knowledge on the nutritive value and phytochemical composition of European varieties of grain legumes and to evaluate the feasibility of including Portuguese varieties of grain legumes in the diet of rainbow trout and Nile tilapia by determining their ADC in both fish species. We successfully achieved the proposed objectives. The main conclusions of this thesis are:

- Several varieties of chickpea, field pea, faba bean and lupins exist well-adapted to Portuguese edaphoclimatic conditions and capable of being sown in Autumn and grown under rainfed conditions, with final grain yields above those traditionally observed;
- Protein content of the studied raw mature grain legume seeds was good varying in average between 22 g/100 g DM (chickpea type Desi var. Elmo and common vetch var. Barril) and ca. 40 g/100 g DM (yellow lupins). Seeds’ starch levels ranged from ca. 27 g/100 g DM in chickpea type Desi var. Elmo to ca. 40 g/100 g DM in common vetch var. Barril and were null in all lupin samples. Neutral detergent fibre and acid detergent fibre were lowest for chickpea type Kabuli varieties (ca. 14 and 3 g/100 g DM, respectively and highest for narrow-leafed lupins (ca. 30 and 20 g/100 DM, respectively);
- Discriminant analysis using seeds crude protein content as the categorical dependent variable showed significant inter-varietal (within grain legume species/groups) differences for some chemical parameters, revealing that the choice for a given variety only based on its high crude protein content may penalize the supply of starch in the

case of field pea and faba beans/common vetch/chickling vetch groups, polyunsaturated fatty acids in the case of chickpeas and total fatty acids in the case of *Lupinus* seeds;

- Polyunsaturated fatty acids predominate in all grain legume species, except in white lupins, where monounsaturated fatty acids prevailed;
- Zeaxanthin contents and therefore total carotenoids were highest for chickpea type Desi var. Elmo. Citric acid and therefore total organic acids were highest for white lupin varieties;
- Phenolics profile of mature raw whole seeds of chickpea type Desi, field peas and common and a further insight into the genotypes of chickpea type Kabuli, faba beans and lupins was achieved for the first time;
- *Lathyrus cicera* seeds were, for the first time, characterized for the phenolics fraction – where the presence of 37 glycosylated flavonoids, mainly kaempferol glycosides was revealed – and evaluated in diets for farmed fish showing good potential, at least at a digestibility level;
- Alkaloids profile of varieties Lumen, Estoril (both white lupins), Nacional (yellow lupin) and Azuro (narrow-leafed lupin) were characterized for the first time;
- Rich-alkaloid lupin extracts showed moderate anti-inflammatory activity, though low antioxidant activity, which could encourage the use of lupins not only as whole seed and flour but as nutraceuticals or therapeutic agents;
- Field pea var. Pixel showed potential in diets for rainbow trout despite its high starch content and chickpeas (var. Elixir and Elmo) in those of Nile tilapia. Need for previous seed processing appear to be only necessary for chickpeas and FB in rainbow trout and for chickling vetch in Nile tilapia to improve overall digestibility of nutrients;
- Overall, the data presented in this dissertation contribute to the valorization of grain legumes as alternative vegetable protein ingredients in compound feedstuffs.

8.3. Future perspectives

- Characterize the protein (albumin and globulin storage proteins) and carbohydrate fractions (mono-, di- and oligosaccharides) of grain legume varieties;
- Characterize grain legume varieties amino acids profiles;
- Determine grain legume varieties minerals and vitamins;

- Complete the characterization of grain legume varieties by determining other phytochemicals in the seeds (e.g. protease and amylase inhibitors, lectins, saponins);
- Characterize the quantitative profile in phenolic compounds of chickling vetch var. Grão-da-gramicha;
- Fully assess the value of grain legumes in aquaculture feed formulations by (1) measuring the ADCs of tested grain legumes at different dietary inclusion levels, (2) performing growth and palatability trials, (3) studying the immune status of fish and (4) determining the effects on the organoleptic quality of the fish;
- Evaluate the potential of including these grain legume varieties also in diets for cattle, pigs and poultry as well as in those of other farmed fish species.

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ANNEXES

ORAL AND IN PANEL COMMUNICATIONS

1 - Poster 1 - Magalhães, S.C.Q., Cabrita, A.R.J., Fonseca, A.J.M. Grain legumes production under rainfed conditions in Portugal. Animal Science Doctoral Programme – I Workshop, 2014, Porto, Portugal.

GRAIN LEGUMES PRODUCTION UNDER RAINFED CONDITIONS IN PORTUGAL

SARA C.Q. MAGALHÃES[1], ANA R.J. CABRITA[1], ANTÓNIO J.M. FONSECA[1]

[1] REQUIMTE/ICBAS, Instituto Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, 4050-313 Porto, Portugal.

ajfonseca@icbas.up.pt

INTRODUCTION

- European Union is strongly dependent on the import of protein sources such as oilseeds and their meals from third countries.
- Several measures have been purposed to increase grain legumes (GL) production in European countries, namely in Portugal (Report A7-0026/2011, 2011).
- Portugal presents potential to produce GL but the actual area occupied by these crops is limited due to a focus of agriculture production on winter cereals, no use of legumes in rotations and low grain yields.

OBJECTIVE

To review the state of knowledge on the Portuguese production of chickpeas, field peas, faba beans and lupins, as these are important GL in Mediterranean farming systems and of interest to the feed industry.

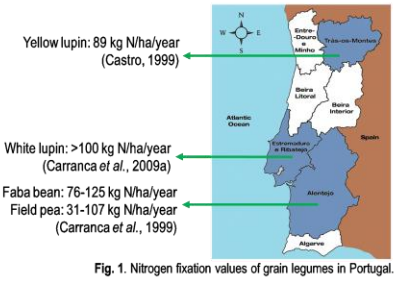

Fig. 1. Nitrogen fixation values of grain legumes in Portugal.

EDAPHOCLIMATIC CONDITIONS

- Portugal is a Mediterranean country with a temperate climate.
- In general, soils are of poor agricultural suitability due to erosion, pH values below 6.5 and reduced levels of organic matter (~ 1%; REA, 1999). Only Entre-Douro e Minho and the alluvial zones of Ribatejo present medium-high OM levels, being allocated to more intensive agricultural systems.

NITROGEN FIXATION

- GL fix nitrogen (N) and store it in root nodules. Data on GL N fixation in Portugal (Fig. 1) agree with those from other Mediterranean countries.

SOWING SEASON

- Portuguese studies on sowing season report that GL perform better in autumn sowing comparing to late winter/spring sowing (Fig. 2), overcoming constrains related with high temperatures/sun irradiation and irregular/scarce rainfall that lead to shortening of growing cycle, low and irregular yields, unsuitable plant habit and consecutively need of manual harvesting.

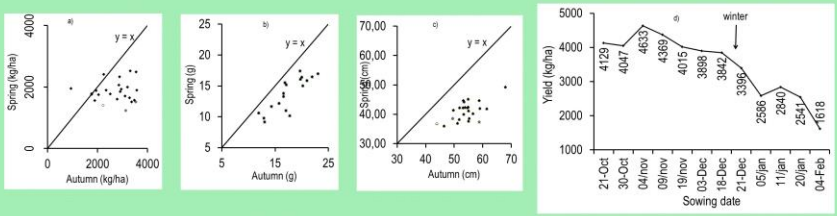

Fig. 2. Effect of sowing season on a) grain yield of chickpea, b) seeds weight of field pea, c) plant habit of chickpea and d) grain yield of faba bean (Costa Pinto *et al.*, 1990; Barroso *et al.*, 2007).

MULTI-SITE YIELD EXPERIMENTS IN AUTUMN SOWING UNDER RAINFED PORTUGUESE CONDITIONS

- National and foreign varities yielded more than what it is traditionally observed (~ 1000 kg/ha).
- Beyond Estremadura e Ribatejo and Alentejo, chickpea performed well in Trás-os-Montes (> 2000 kg/ha).
- Field pea high yields in all regions (Fig. 3) suggest its adaptation to a wide range of agronomic conditions.
- Sandy, acidic, low fertile soils of Alentejo may be responsible for the low grain yields reported for some sweet white lupins.

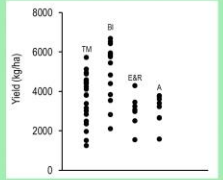

Fig. 3. Grain yield (kg/ha), in autumn/ winter sowing, in Portugal, of foreign or Portuguese varieties not included in the National Catalogue of varities of field peas. (Dordio, 1990; Barroso *et al.*, 2007; Reis and Rodrigues, 2010)

CEREAL-BASED ROTATIONS

- Rainfed cereals are sown in autumn/winter in N poor fields, commonly in rotation with fallow (NOT recommended!).
- Rotations with GL can greatly contribute to the development of the following cereal (Fig. 4 and Carranca *et al.*, 2009b) by restoring the N/other nutrients in the soil and decreasing the incidence of pests/diseases in cereals and GL.

ECONOMICAL AND SUSTAINABLE FARMING PRACTICES

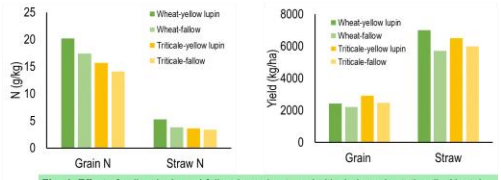

Fig. 4. Effect of yellow lupin and fallow in a wheat- and triticale-based rotation (in Alentejo, Portugal) on cereals' grain and straw nitrogen (N) content and yield (Castro, 1999).

CONCLUSIONS

- Several GL varieties are well-adapted to our edaphoclimatic conditions, capable of being sown in autumn and grown under rainfed conditions, with final grain yields above those traditionally observed.
- Grain legumes-based rotations, replacing the traditional fallow, could benefit the cereal and contribute to the development of organic and more extensive farming systems, leading to higher farmer incomes while helping to combat human desertification in North and Central inland, and South of the country.

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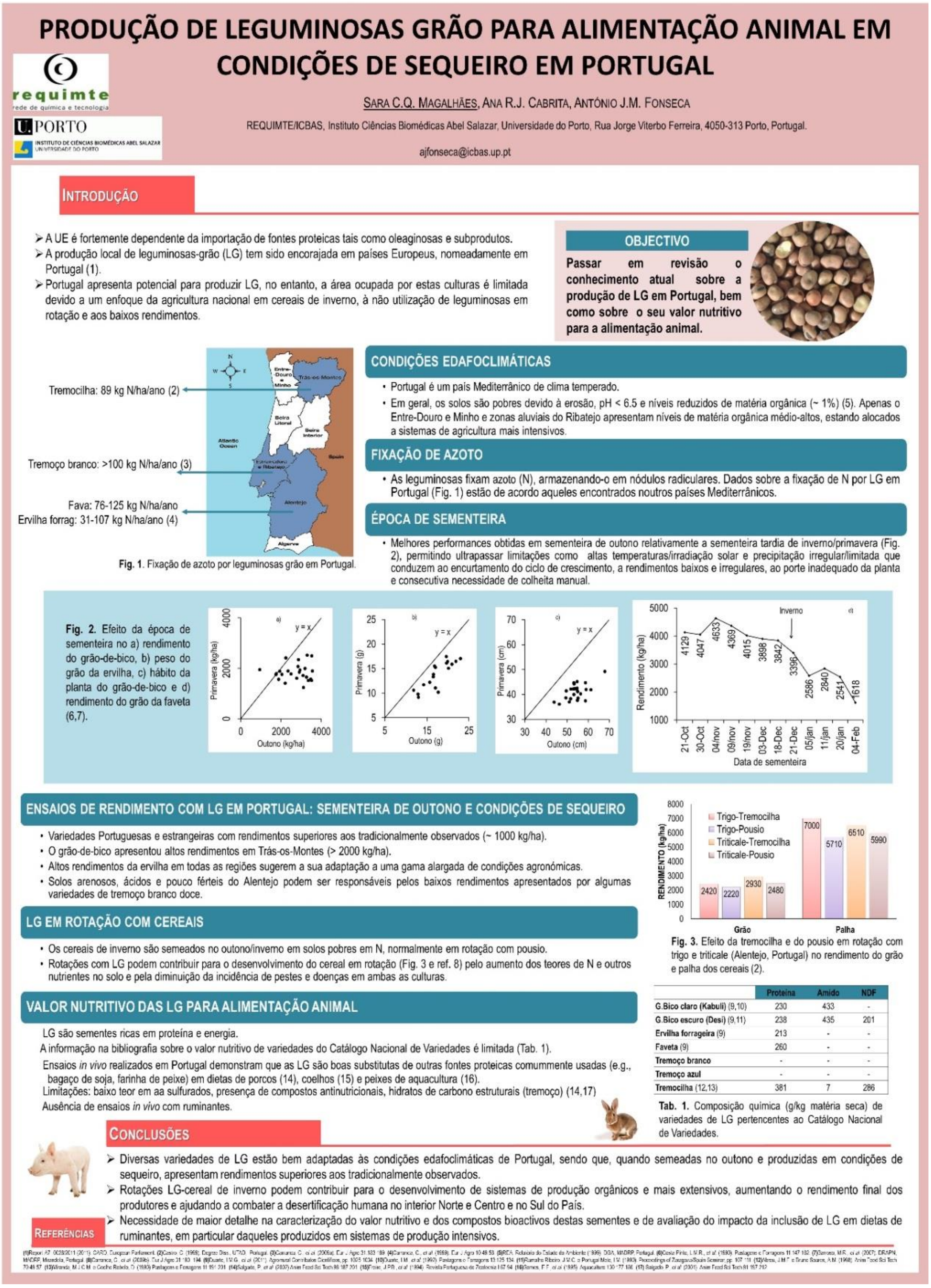
2 - Poster 2 - Magalhães, S.C.Q., Cabrita, A.R.J., Fonseca, A.J.M. Produção de proteaginosas grão para alimentação animal em condições de sequeiro em Portugal. Encontro de Primavera da Sociedade Portuguesa de Pastagens e Forragens, Vila Pouca de Aguiar, 2015, Vila Real, Portugal.



3 – Poster 3 - Magalhães, S.C.Q., Cabrita, A.R.J., Valente, L.M.P., Rema, P., Fonseca, A.J.M. Apparent digestibility coefficients of Portuguese grain legumes in rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*). Animal Science Doctoral Programme – II Workshop, 2015, Porto, Portugal.

APPARENT DIGESTIBILITY COEFFICIENTS OF PORTUGUESE GRAIN LEGUMES IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AND NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

Sara C.Q. Magalhães[1]
Ana R.J. Cabrita[1]
Luisa M.P. Valente[1,2]
Paulo Rema[3]
António J.M. Fonseca[1]

[1]REQUIMTE/LAQV, ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, 4050-313 Porto, Portugal

[2]CIIMAR-CIMAR L.A., Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal

[3]Universidade de Trás-os-Montes e Alto Douro (UTAD), Quinta dos Prados, P.O. Box 1013, 5001-801 Vila Real, Portugal

E-mail: ajfonseca@icbas.up.pt

Introduction

- Looking for fish meal (FM) replacers is a major international research priority driven by the declining supply of FM and the expanding of aquaculture.
- Grain legumes (GL), capable of being produced in Europe, are low-price ingredients and offer a certain flexibility to the feed manufacturer as they might replace both grain and protein sources (Cruz-Soarez *et al.*, 2001).

Objective

- To determine the apparent digestibility coefficients (ADC) of dry matter (DM), crude protein (CP), ether extract (EE) and gross energy (GE) of Portuguese varieties of GL in rainbow trout and Nile tilapia, important aquaculture species with distinct feeding habits (carnivorous and omnivorous, respectively).

Methods

- GL included Kabuli (**CHK**) and Desi (**CHD**) types of chickpea (*Cicer arietinum*), field pea (**FP**, *Pisum sativum*), faba bean (**FB**, *Vicia faba* minor), white lupin (**WL**, *Lupinus albus*) and chickling vetch (**CV**, *Lathyrus cicera*).
- For each fish species, 70% of the reference diet (**REF**), previously extruded, was added to 30% of each raw GL (ground to 0.5 mm). Chromic oxide was used as inert marker at 1% in the diet (Cho and Slinger, 1979). Diets were pelleted dry without steam (4 mm).
- Fish feeding and rearing conditions were as described by Pereira *et al.* (2012) and faeces were collected using the Choubert system (Choubert *et al.*, 1982).
- ADCs (of nutrients and energy) of diets and ingredients were calculated according to Maynard *et al.* (1979) and Bureau *et al.* (1999), respectively.

Results

- Protein ADCs of experimental diets were similar or above ($P < 0.05$) those of the respective REF diet (Table 1), suggesting these ingredients as potential replacers of FM.

RAINBOW TROUT

- FP diet showed the highest CP ADC (Table 1).
- 30% of CHK, CHD and FB decreased ($P < 0.05$) DM and GE ADCs to values below the REF diet (67-73%). Also, the DM and GE ADCs of the ingredients themselves were low (52-58%), agreeing with previous results on perch (Booth *et al.*, 2001). Higher ADCs could be achieved with seeds dehulled, extruded or in a protein concentrate form.
- FP presented overall higher ($P < 0.05$) ADCs, but similar to those of WL and CV. Despite FP lower protein content than WL, higher fiber levels of WL and also of CV may compromise their use in fish diets comparing to FP.

Table 1. ADCs (%) of protein (CP) and energy (GE) of reference and experimental diets.

ADC diets		REF	CHK	CHD	FP	FB	WL	CV	*P*
CP	Trout	90.6^{a}	$91.1^{a,b}$	90.6^{a}	$93.3^{b,c}$	90.2^{a}	$91.7^{a,c}$	$90.6^{a,c}$	0.014
	Tilapia	$85.7^{a,c}$	$88.2^{a,b}$	$88.2^{a,b,d}$	$87.9^{a,b}$	91.0^{d}	$89.5^{b,d}$	84.5^{c}	< 0.001
GE	Trout	79.3^{a}	71.8^{b}	71.8^{b}	81.9^{a}	$73.0^{b,c}$	$77.8^{a,c}$	$79.3^{a,b}$	0.001
	Tilapia	69.2^{a}	$77.7^{b,c}$	75.3^{c}	70.6^{a}	80.4^{b}	$76.0^{b,c}$	70.2^{a}	< 0.001

NILE TILAPIA

- FB diet showed the highest CP ADC (Table 1).
- ADCs of DM and GE were higher ($P < 0.05$) for FB and CHK diets, the values for REF, FP and CV diets being the lowest (ca. 70%). The ADC of EE was lowest for REF and FP diets (81% vs. 87-90%).
- FB and CHK had the highest ADCs for DM and GE (94-108%). Overall lower ADCs were found for CV diet and for the ingredient itself which may be related with high levels of condensed tannins, phytic acid and neurotoxin β-ODAP in the seeds (Ramachandran and Ray, 2008).

INTERACTION FISH × GL ON ADCs

- While trout seemed to digest better FP and CV, tilapia did better with the other GL (Figure 1).
- With feeding habits based on vegetable material, tilapia more easily digests carbohydrates (starchy and fibrous) and possible antinutritional compounds present in GL than trout (carnivorous).
- The choice between Kabuli and Desi chickpea types may be taken in accordance with price and availability of seeds, as identic ADCs were obtained.

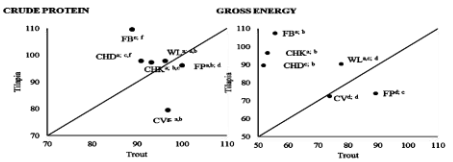


Figure 1. Interaction fish × GL on the ADCs (%) of CP and GE. Different superscript letters indicate significant differences in the ADCs between fish species at $P < 0.05$ (the letter(s) before semicolon correspond(s) to tilapia and the letter(s) after semicolon to trout).

Conclusions

- Both digestibility trials revealed Portuguese GL with potential to be included in aquafeeds.
- Increased DM and GE ADCs of both chickpeas and FB in trout and of CV in tilapia may be achieved after seeds processing to overcome issues related to indigestible carbohydrates and antinutritional factors.
- This was the first report on the use of CV in trout and tilapia and on the use of chickpeas in tilapia.

Acknowledgements
This work received financial support from the European Union (FEDER funds through COMPETE) and National Funds (FCT) through UID/QUI/50006/2013 (LAQV). S.C.Q. Magalhães is indebted to FCT for the grant (SFRH/BD/80783/2011). The authors also acknowledge INIAV, I.P. for supplying the GL samples and Universidade de Trás-os-Montes e Alto Douro (UTAD) for the fishes and facilities necessary to develop the trials.

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4 - Communication 1 - Magalhães, S.C.Q., Cabrita, A.R.J., Taveira, M., Valentão, P., Andrade, P.B, Fonseca, A.J.M.,. Mediterranean grain legumes: chemical composition and bioactive compounds. Animal Science Doctoral Programme – I Workshop, 2014, Porto, Portugal.

MEDITERRANEAN GRAIN LEGUMES: CHEMICAL COMPOSITION AND BIOACTIVE COMPOUNDS

Sara C. Q. Magalhães*[1], Ana R. J. Cabrita[1], Marcos Taveira[2], Patrícia Valentão[2], Paula B. Andrade[2], António J. M. Fonseca[1]

[1]REQUIMTE/ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, 4050-313 Porto, Portugal.
[2]REQUIMTE/Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 4050-313 Porto, Portugal.
E-mail: ajfonseca@icbas.up.pt

Introduction

Grain legumes (GL) are crops of the botanical family Fabaceae grown for food and feed. Their recognized high protein content is the reason why they are dubbed "poor man's meat" in low-income groups in developing countries (Tharanathan and Mahadevamma, 2003). In animal nutrition, GL also constitute appealing economical and sustainable alternatives to the protein sources commonly used (for instance soybean meal; e.g. Jezierny *et al.*, 2010). Mediterranean countries present suitable edaphoclimatic conditions for GL growth and measures towards increasing GL production have already been purposed in the European Union. Beyond proteins, legume seeds are great sources of energy and fiber (Jezierny *et al.*, 2010) also containing non-nutrient bioactive compounds that may exert positive, negative or both effects in those who ingest them (Champ, 2002). For instance, while carotenoids and phenolic compounds confer antioxidant properties to GL, other metabolites such as oxalates, enzyme inhibitors or alkaloids may decrease nutrients' availability and digestibility in the gastrointestinal tract. Therefore, it seems imperative to determine in detail the nutritive value of GL available to better include these ingredients in humans' and animals' diets. The aim of this work was to characterize several Mediterranean GL varieties regarding nutritional and bioactive properties. Whenever possible, Portuguese (PT) varieties were compared with those from foreign (F) countries.

Species chosen and analysis

Grain legumes studied included Kabuli (beige; n=5) and Desi (dark; n=1) chickpea (*Cicer arietinum*), field pea (*Pisum sativum*; n=21), faba bean (*Vicia faba* var. minor; n=10), white (n=5), narrow-leafed (n=2) and yellow (n=5) lupins (*Lupinus albus*, *L. angustifolius* and *L. luteus*, respectively), chickling vetch (*Lathyrus cicero*; n=1) and common vetch (*Vicia sativa*; n=1) which were provided by several companies from Portugal, Spain, France, Italy and Poland. Fourteen varieties belonged to the Portuguese Catalog of Varieties. Proximate composition was determined in all varieties as described by Cabrita *et al.* (2011). Seeds were also analyzed for fatty acids profile by GC, according to Alves *et al.* (2008), organic acids by HPLC-UV following Sousa *et al.* (2009), carotenoids by HPLC-DAD as described by Mariutti *et al.* (2012) and phenolic compounds by HPLC-DAD according to Silva *et al.* (2005).

Major results

Among all samples, protein content ranged between 21.0 and 42.8% DM with values above 32% belonging to lupins. Protein fraction was characterized as being highly soluble (62.7±5.41%) in all samples. Fattest samples were chickpeas and lupins (4.7±0.83 and 6.2±1.74% ether extract in DM, respectively) and the major fatty acids found in all varieties were palmitic (16:0), oleic (18:1c9) and linoleic (18:2) acids that accounted for more than 75% of total fatty acids. Within chickpeas, the dark variety, which is suited for animal feeding, presented less fat and starch contents and higher levels of cell-wall components than beige seeds. Starch content ranged from 27.3 to 44.6% DM in all samples, lupins being an exception. Indeed, lupins lacked starch but presented increased amount of non-starch polysaccharides (17-29% DM) comparatively to the other samples. Major differences between PT and F varieties were observed in beige chickpeas; PT seeds presented (DM basis), in average, less 4.5 percent points (pp) of protein and more 1.4 pp of fat and 5.4 pp of starch, relatively to F ones. Also, PT faba beans and white lupins had lower protein content, while field peas and white lupins showed similar values between both groups.

Among all varieties, only two carotenoids were identified, namely lutein and zeaxanthin. Lutein was present in all samples and zeaxanthin only in chickpea and lupins. Of all chickpeas, dark variety stood out from the beige ones in terms of total carotenoids content (162.3 vs. 28.7-87.6 μg g^{-1} DM, respectively).

Indeed, dark chickpeas present higher antioxidant activity (Segev *et al.*, 2010), carotenoids contributing for that. Main differences between PT and F varieties were also found in beige chickpeas, with the formers presenting higher total carotenoids levels.

Several organic acids were identified in GL seeds (Figure 1). Citric and aconitic acids (antioxidant agents) were common to all varieties, the former being the major compound in all samples. Lupins presented the highest total amount of organic acids (4.0±0.43 mg g^{-1} DM) and common vetch the lowest (0.5 mg g^{-1} DM). Among all, oxalic acid should be highlighted once it affects calcium and magnesium metabolism and protein digestion when ingested mainly by monogastrics (Akande *et al.*, 2010). Results showed field peas and faba beans to lack oxalic acid and the other species to contain between 2.0 and 7.7 mg 100 g^{-1}. These values are considered low for human consumption (OHF, 2008) and are below those found for soybean seeds (Massey *et al.*, 2001). PT yellow lupin presented higher organic acids content than F ones mainly due to increased citric acid levels.

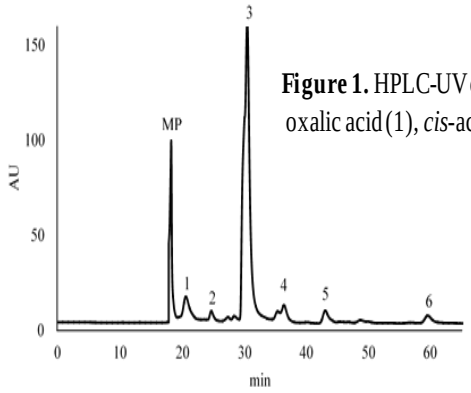

Figure 1. HPLC-UV organic acids profile of chickpea var. Elvar. Peaks identification: mobile phase (MP), oxalic acid (1), *cis*-aconitic acid (2), citric acid (3), malic acid (4), *trans*-aconitic acid (5) and fumaric acid (6).

With the exception of chickpea samples, in which no phenolic compounds were detected, phenolic acids and flavones were the metabolites identified in GL seeds. The profile quietly varied between species and in some cases within varieties of the same species. Samples with a higher content and a more detailed profile in phenolic compounds were field peas (0.15-0.44 mg g^{-1} DM) and faba beans (0.30-0.41 mg g^{-1} DM).

Conclusions

Although for some species, PT varieties were not as proteinaceous as F ones, they all represent good sources of protein, energy and unsaturated fatty acids for humans and animals. The content of xantophylls, citric acid and phenols is indicative of the antioxidant power of these seeds in biological systems. Oxalates do not constitute a problem in any of the samples studied.

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5 - Communication 2 - Magalhães, S.C.Q., Cabrita, A.R.J., Taveira, M., Valentão, P., Andrade, P.B., Fonseca, A.J.M. Mediterranean grain legumes: a source of bioactive compounds. XX Encontro Luso-Galego, 2014, Porto, Portugal.

Mediterranean grain legumes: a source of bioactive compounds

Sara C. Q. Magalhães[1], Ana R. J. Cabrita[1], Marcos Taveira[2], Patrícia Valentão[2], Paula B. Andrade[2], António J. M. Fonseca[1]

[1]REQUIMTE/ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal.
[2]REQUIMTE/Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal.
E-mail: ajfonseca@icbas.up.pt

Grain legumes (GL) play an important role in the humans' diet and constitute appealing economical and sustainable alternatives to the protein sources commonly used in animal feeding (e.g. soybean meal) [1]. Beyond proteins, legume seeds are great sources of energy and fiber [1] and also contain non-nutrient bioactive compounds that may exert positive, negative or both effects in those who ingest them. Thus, the aim of this work was to increase the knowledge on the bioactive compounds of several Mediterranean GL varieties regarding their utilization in human and animal nutrition. In this study, Kabuli (beige; n=5) and Desi (dark; n=1) chickpeas (*Cicer arietinum*), field peas (*Pisum sativum*; n=6), faba beans (*Vicia faba* var. *minor*; n=6), white (n=4), narrow-leafed (n=2) and yellow (n=4) lupins (*Lupinus albus*, *L. angustifolius* and *L. luteus*, respectively), chickling vetch (*Lathyrus cicera*; n=1) and common vetch (*Vicia sativa*; n=1) were evaluated. These samples were provided by companies from Portugal, France, Italy and Poland. Fourteen varieties belonged to the Portuguese Catalog of Varieties. Seeds were analyzed for organic acids by HPLC-UV [2] and for carotenoids [3] and phenolic compounds by HPLC-DAD [4].

Concerning to the carotenoids profile, among all varieties, only two carotenoids were identified, namely lutein and zeaxanthin. Lutein was present in all samples and zeaxanthin only in chickpea and lupins. Of all chickpeas, the dark variety (suited for animal feeding) stood out from the beige ones in terms of total carotenoids content (162.3 *vs.* 28.7-87.6 µg/g dry matter, DM, respectively). Indeed, it was already reported that dark chickpeas present higher antioxidant activity [5], for which carotenoids may give a contribution. Portuguese beige chickpeas presented higher total carotenoids levels. Several organic acids were identified in GL seeds. Citric and aconitic acids (antioxidant agents) were common to all varieties, the former being the major compound in all samples. Lupins presented the highest total amount of organic acids (4.0±0.43 mg/g DM) and common vetch the lowest one (0.5 mg/g DM). Among all, oxalic acid should be highlighted once it affects calcium and magnesium metabolism and protein digestion when ingested mainly by monogastrics [6]. Results showed field peas and faba beans to lack oxalic acid and the other species to contain between 2.0 and 7.7 mg/100 g. These values are considered low for human consumption [7] and are below those found for soybean seeds [8]. The Portuguese yellow lupin presented higher organic acids content than foreign ones mainly due to increased citric acid levels. In terms of phenolic compounds, they were not detected in all chickpeas, one white lupin and one faba bean varieties studied. In all the other samples, phenolic acids and flavones were the metabolites identified. Several differences between species were noticed and in some cases also within varieties of the same species. Field peas and faba beans presented a bigger variety and amount (0.35-0.45 mg/g DM) of phenolic compounds. In conclusion, the GL analysed are a good source of bioactive compounds, namely carotenoids, citric acid and phenolics, which are well known by their health promoting effects. In addition, oxalates do not constitute a problem in any of the samples studied. In this matter, Portuguese varieties seem promising options for both human and animal nutrition.

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> Química Agro-Mar-Alimentar

6 - Communication 3 - Magalhães, S.C.Q., Fernandes, F., Andrade, P.B., Valentão, P., Cabrita, A.R.J., Fonseca, A.J.M. Perfil em alcaloides de variedades mediterrânicas de *Lupinus albus*, *L. angustifolius* e *L. luteus*. XIX Congresso de Zootecnia, 2015, Ponte de Lima, Viana do Castelo, Portugal.

COMPOSIÇÃO EM ALCALOIDES DE VARIEDADES MEDITERRÂNICAS DE *LUPINUS ALBUS*, *L. ANGUSTIFOLIUS* E *L. LUTEUS*

Magalhães[1], S.C.Q., Fernandes[2], F., Andrade[2], P.B., Valentão[2], P., Cabrita[1], A.R.J., Fonseca[1], A.J.M.

[1]REQUIMTE/ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal. [2]REQUIMTE/LAQV, Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal.
E-mail: ajfonseca@icbas.up.pt

INTRODUÇÃO

A indústria dos alimentos para animais enfrenta na Europa uma crise relacionada com a falta de fontes proteicas de origem vegetal. A maior parte dos ingredientes proteicos utilizados é importada de países terceiros (exemplo da soja e seus subprodutos), tendo um forte impacto negativo na balança comercial dos países e na rentabilidade das explorações. Neste contexto, a Comissão Europeia tem vindo a desenvolver esforços no sentido de incentivar a produção de plantas ricas em proteína, dando especial enfoque a leguminosas-grão (LG), como grão-de-bico, ervilha, fava, entre outras, por apresentarem elevados níveis de proteína bruta (PB) (20-38% na matéria seca, MS) e um perfil nutricional interessante para a alimentação animal. Entre diversas LG, as sementes de tremoceiros têm a vantagem de apresentar níveis mais elevados de PB (32-38% na matéria seca; Petterson, 2000), sendo, por isso, amplamente valorizadas como alternativas aos ingredientes proteicos atualmente usados. No entanto, como todas as LG, os tremoceiros apresentam compostos antinutricionais, resultantes do metabolismo secundário, tais como fitolectinas, saponinas, oligossacarídeos, ácido fítico e alcaloides (Muzquiz *et al.*, 2012). Estes últimos, em particular, são os compostos antinutricionais mais abundantes nos tremoceiros e com efeitos toxicológicos mais importantes, nomeadamente no sistema nervoso central, nos processos digestivos e nos sistemas reprodutor e imunitário, principalmente de animais monogástricos como aves e suínos (Pastuszewska *et al.*, 2001) e peixes (Glencross *et al.*, 2006). Os alcaloides podem ainda diminuir a palatabilidade das dietas, afetando, por consequência, a ingestão voluntária do alimento. No entanto, a inclusão de diferentes espécies de tremoceiros em dietas de aves, porcos, ruminantes e peixes foi já descrita com sucesso (e.g. Barneveld, 1999). Os alcaloides presentes nas sementes de tremoceiros derivam do aminoácido lisina e compreendem os alcaloides quinolizidínicos (lupanina, angustifolina, esparteína, entre outros), presentes em elevadas quantidades, e, por vezes, alcaloides piperidínicos (piperina) e indólicos (gramina) (Koleva *et al.*, 2012). Diversas técnicas de processamento podem ser aplicadas de forma a reduzir o teor em alcaloides dos tremoceiros, nomeadamente a sua embebição em água, o descascamento e a fervura. Existem, no entanto, variedades com teor muito reduzido ou inexistente de alcaloides (apelidadas de "variedades doces"), resultantes de trabalhos de melhoramento genético.

Para melhor selecionar sementes de tremoceiros para uso animal, as variedades disponíveis devem ser caracterizadas ou monitorizadas relativamente à sua composição em alcaloides. Neste sentido, o objetivo do presente trabalho foi o de determinar o perfil qualitativo e quantitativo destes compostos em variedades mediterrânicas de tremoceiros.

MATERIAL E MÉTODOS

Foram analisadas doze variedades de tremoceiros que compreendiam tremoço branco (*Lupinus albus*; n=5), tremoço azul (*L. angustifolius*; n=2) e tremocilha (*L. luteus*; n=5), gentilmente cedidas por empresas de Portugal, França e Itália (Quadro 1). Foram também analisadas variedades polacas já cultivadas com sucesso em Itália (Gresta *et al.*, 2010). Após receção, as sementes foram secas em estufa com circulação forçada de ar (65ºC, 24 h) e moídas (1 mm). A extração dos alcaloides realizou-se de acordo com Muzquiz *et al.* (1994) e Gresta *et al.* (2010). Sucintamente, 2 g de amostra foram homogeneizadas em 20 ml de ácido tricloroacético 5% durante 30 min, a 400 rpm, e centrifugadas a 4000 rpm por 15 min. O processo foi repetido por duas vezes. Os sobrenadantes foram reunidos e adicionaram-se 4 ml de hidróxido de sódio 10 M. Foi feita uma extração líquido-líquido com

diclorometano. O extrato foi evaporado até à secura (sob vácuo num evaporador rotativo) e os alcaloides diluídos em volume apropriado de diclorometano. A cafeína (1 mg/ml) foi usada como padrão interno. Procedeu-se à identificação dos alcaloides presentes nos extratos por cromatografia gasosa acoplada a espetrometria de massa (CG-EM) e à sua quantificação por CG acoplada a uma detetor de ionização de chama (FID). As condições de separação cromatográfica foram as descritas por Gresta *et al.* (2010). Os alcaloides foram identificados por comparação do seu índice de retenção e espetro de massa com padrões externos (gramina, lupanina e angustifolina) e com dados bibliográficos (Wink *et al.*, 1995; WebBook, http://webbook.nist.gov/chemistry/). A quantificação dos compostos foi, por sua vez, realizada pelo método do padrão externo usando retas de calibração. Os compostos esparteína, α-isolupanina, 11,12-desidrolupanina e 13-hidroxilupanina foram quantificados como lupanina. Os restantes foram quantificados como eles mesmos.

RESULTADOS E DISCUSSÃO

Foram identificados sete alcaloides nas amostras de tremoceiros: gramina, esparteína, angustifolina, α-isolupanina, lupanina, 11,12-desidrolupanina e 13-hidroxilupanina (Quadro 1, Figura 1). Todos os alcaloides encontrados são quinolizidínicos, com a exceção da gramina (indólico). Como é normalmente dado maior enfoque aos alcaloides quinolizidínicos, a informação relativa à gramina é escassa. No entanto, este metabolito tem sido, juntamente com a lupinina e a esparteína, alvo de estudo, principalmente em animais monogástricos, por ser dos alcaloides com maior toxicidade, estando descritas concentrações máximas de inclusão em dietas de aves, porcos e peixes (Pastuszewska *et al.*, 2001; Glencross *et al.*, 2006). De acordo com Wink *et al.* (1995) poucas espécies de tremoceiros produzem gramina, sendo típico das tremocilhas (Petterson, 2000). No entanto, no presente trabalho, a gramina foi encontrada, a par da esparteína, em tremocilha e também em três variedades de tremoço branco; no entanto, em quantidades negligíveis. Apesar da lupanina ser descrita como o alcaloide maioritário em tremoceiros, não foi detetada na tremocilha. No entanto, resultados semelhantes foram já obtidos por Gresta *et al.* (2010). Apesar da angustifolina não ser comum em tremoço branco, este composto foi identificado em algumas variedades, tal como previamente descrito (Wink *et al.*, 1995; Gresta *et al.*, 2010).

O perfil quantitativo de alcaloides nas amostras apresentou variação inter e intraespecífica (Quadro 1). A lupanina foi o composto maioritário nas variedades dos tremoceiros branco e azul e a esparteína nas de tremocilha, indo de encontro ao previamente descrito por diversos autores (e.g. Boschin *et al.*, 2008; Gresta *et al.*, 2010). No entanto, de forma geral, os valores encontrados são superiores aos descritos. Todas as variedades de tremoceiro branco, com a exceção da Multitalia, apresentaram um teor total em alcaloides inferior a 50 mg/100g MS, sendo, por isso, consideradas variedades doces (Pilegaard e Gry, 2009). Os elevados teores de lupanina verificados para a variedade Multitalia (1.40-4.62 g/100g MS) não eram expectáveis, uma vez que esta é descrita na literatura (Andrada *et al.*, 2008; Gresta *et al.*, 2010), e pelo próprio fornecedor de semente, como uma variedade isenta ou com baixo teor de alcaloides. Por outro lado, é também referida como uma variedade antiga/histórica e com menor seleção genética comparativamente a outras (Calabrò *et al.*, 2015). A explicação para valores tão elevados e para a diferença no teor total de alcaloides entre ambas as variedades Multitalia (com diferentes origens) poderá dever-se, para além de motivos relacionados com melhoramento genético, a diferentes e/ou menos favoráveis condições ambientais a que a planta foi sujeita durante o crescimento (Christiansen *et al.*, 1997; Calabrò *et al.*, 2015). Relativamente às tremocilhas, Dukat apresentou níveis negligíveis de alcaloides, sendo, assim como Mister, considerada variedade doce. Por outro lado, Taper e Nacional são variedades amargas (> 50mg/100g MS). Gresta *et al.* (2010) descreveu resultados semelhantes para Dukat mas valores inferiores para Mister (0.9 mg/100g) e Taper (1.4 mg/100g). Ambas as variedades de tremoceiro azul apresentaram niveis altos de angustifolina (9.7-383.1 mg/100g MS) e lupanina (52.2-1996.9 mg/100g MS), sendo, por isso, variedades amargas.

Das variedades doces identificadas (< 50 mg/100g MS; Quadro 1), apenas Estoril, Amiga e ambas as variedades Mister podem ser incluídas na alimentação animal sem qualquer restrição uma vez que apresentam um teor de alcaloides inferior ao limite de segurança imposto por autoridades do Reino Unido, França e Austrália (20 mg/100g MS; Pilegaard e Gry, 2009). Os valores elevados de lupanina e esparteína encontrados em Multitalia, Nacional, Taper e Azuro podem condicionar o seu uso em alimentação de suínos por estarem acima das doses letais descritas para esta espécie animal (Kim *et al.*, 2007). Os suínos são, comparativamente com as aves, animais mais sensiveis a alcaloides de tremoço (Berneveld, 1999; Petterson, 2000). Também não parece aconselhável incluir qualquer uma das variedades de tremocilha (exceto Dukat) em dietas de peixes por apresentarem um teor em esparteína superior a 10 mg/100g MS (Serrano *et al.*, 2012). Em herbívoros, a lupanina e esparteína apresentam toxicidade média (Wink *et al.*, 1995), no entanto, naqueles que são ruminantes, a presença de qualquer composto antinutricional é de menor importância relativamente a monogástricos, uma vez que as reações que ocorrem no rumen são capazes de transformar esses mesmos compostos em formas menos tóxicas (Dixon and Hosking, 1992).

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Quadro 1. Perfil e composição (mg/100g de matéria seca) em alcaloides de variedades mediterrânicas de tremoço.

Amostra	Origem	1	2	3	4	5	6	7	Total
Tremoço branco									
Lumen	França	nq	nq	3.1	nq	27.8	nd	nq	30.9
Estoril	Portugal	nq	nq	nq	nq	19.8	nq	nq	19.8
Multitalia	Portugal	nd	nd	nq	nd	1401.5	nd	nd	1401.5
Multitalia	Itália	nd	nd	74.7	nd	4623.7	nd	nd	4698.4
Amiga	França	nq	nq	nd	nq	15.5	nd	nq	15.5
Tremocilha									
Dukat	Polónia	nq	nq	nq	nd	nd	nd	nd	0.0
Nacional	Portugal	nq	623.3	nd	nd	nd	nq	nd	623.3
Mister	Portugal	nq	20.8	nq	nd	nd	nq	nd	20.8
Mister	Polónia	nq	13.9	nd	nd	nd	nq	nd	13.9
Taper	Polónia	nq	53.8	nd	nd	nd	nd	nd	80.3
Tremoço azul									
Azuro	Portugal	nd	nd	383.1	nq	1996.9	nd	nd	2380.0
Sonet	Polónia	nd	nd	9.7	nq	52.2	18.4	nd	80.3

(1) Gramina, (2) Esparteína, (3) Angustifolina, (4) α-Isolupanina, (5) Lupanina, (6) 11,12-Desidrolupanina, (7) 13-Hidroxilupanina. nd, não detetado (valor inferior ao limite de deteção: gramina, 0.05 mg/ml; angustifolina, 0.02 mg/ml; lupanina, 0.08 mg/ml); nq, não quantificado (valor inferior ao limite de quantificação: gramina, 0.16 mg/ml; angustifolina, 0.05 mg/ml; lupanina, 0.24 mg/ml).

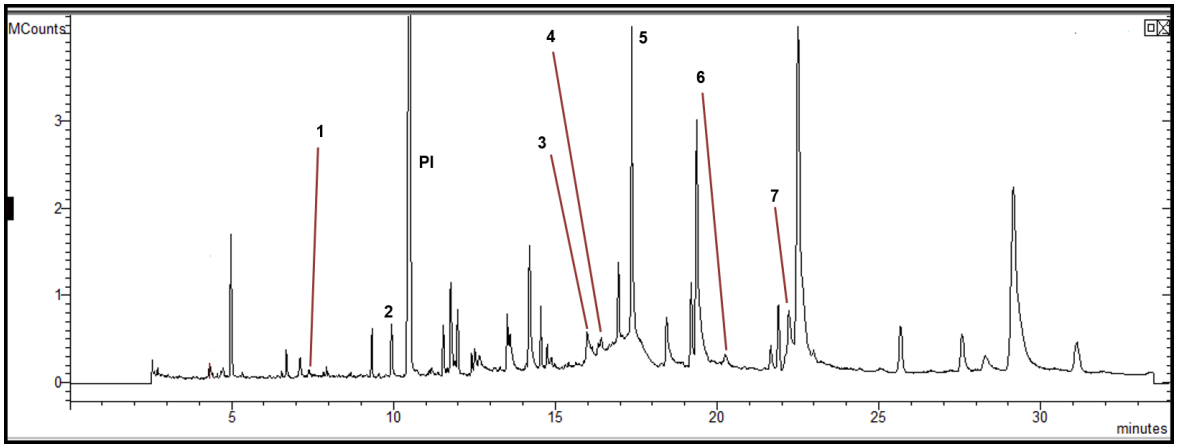

Figura 1. Exemplo geral do perfil cromatográfico obtido por CG-EM de alcaloides de tremoço branco (*Lupinus albus*). Identidade dos compostos de acordo com o Quadro 1. PI, padrão interno (cafeína).

ALKALOIDS PROFILE OF MEDITERRANEAN VARIETIES OF *LUPINUS ALBUS*, *L. ANGUSTIFOLIUS* AND *L. LUTEUS*

ABSTRACT

In a European scenario of external dependence on protein crops for animal feeding, lupins (*Lupinus* spp.) are suggested as valuable alternatives to the commonly used vegetable protein ingredients, as they supply 32-38% of protein (dry matter basis, DM). However, lupins contain alkaloids as main antinutritional factors, which may cause several toxic effects on animals. In the present work, the qualitative and quantitative alkaloids profile of twelve mediterranean varieties of different lupins species (*L. albus*, white lupin; *L. angustifolius*, narrow-leafed lupin; *L. luteus*, yellow lupin) was determined by GC. Seven alkaloids were identified, namely, gramine, sparteine, angustifoline, α-isolupanine, lupanine, 11,12-dehydrolupanine and 13-hydroxylupanine. The major compounds found were lupanine in white (16-4624 mg/100g DM) and narrow-leafed lupins (52-1997 mg/100g DM) and sparteine in yellow lupins (14-623 mg/100g DM). Six varieties comprising white and yellow lupins were considered sweet (< 50 mg/100g DM). The high contents of lupanine and sparteine obtained for some varieties may compromise their use in pig nutrition. Also, sparteine levels of yellow lupins above 10 mg/100g MS may limit their inclusion in aquafeeds. Major focus of attention must, therefore, be given to the sweet varieties also taking in account the individual composition of lupins alkaloids in order to better include these seeds in animal feeding.

Keywords: Alkaloids, Gas chromatography, Lupins, mediterranean

7 - Communication 4 - Magalhães, S.C.Q., Fernandes, F., Cabrita, A.R.J., Fonseca, A.J.M., Valentão, P., Andrade, P.B. Alkaloids profile of European lupin seeds (*Lupinus* spp.) used in food and feedstuffs. Animal Science Doctoral Programme – II Workshop, 2015, Porto, Portugal.

ALKALOIDS PROFILE OF EUROPEAN LUPIN SEEDS (*LUPINUS* SPP.) USED IN FOOD AND FEEDSTUFFS

Sara C. Q. Magalhães[1]*, Fátima Fernandes[2], Ana R. J. Cabrita[1], António J. M. Fonseca[1], Patrícia Valentão[2], Paula B. Andrade[2]

[1] REQUIMTE/LAQV, ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, 4050-313 Porto, Portugal
[2] REQUIMTE/LAQV, Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, R. Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal.
E-mail: pandrade@ff.up.pt

Introduction

Lupin seeds (*Lupinus* spp.) are low price and non-genetic modified ingredients that constitute good sources of protein (ca. 40%), fiber (ca. 28%), healthy fatty acids, vitamins, minerals and other metabolites with recognized antioxidant properties (e.g., polyphenols). However, they contain alkaloids as main antinutritional factors, which may cause several types of disorders on humans and animals. Considering the recent efforts towards increasing the local production of protein-rich crops, with emphasis on lupins, in the European countries for food and feed purposes, the present work aimed at determining the alkaloids profile of some lupins grown in Mediterranean countries and in Poland. The potential of the studied lupin seeds to be included in food and feed is here briefly discussed, based on our results on seeds alkaloids composition and on relevant information available in the literature.

Material and methods

Eleven varieties (included in the European Plant Variety Database) and one Portuguese ecotype of lupins, corresponding to mature raw seeds of *L. albus* (white lupin, WL; n=5), *L. angustifolius* (narrow-leafed lupin, NLL; n=2) and *L. luteus* (yellow lupin, NLL; n=2), were analyzed. Seeds were dried (65 ºC, 24 h), ground (1 mm) and dry matter (DM) content determined after drying the powdered samples at 103 ºC overnight. Alkaloids were extracted as previously described by Muzquiz *et al*. (1994) and Gresta *et al*. (2010), with slight modifications. Alkaloids identification and quantification in the rich-alkaloid extracts was performed by GC-MS and GC-FID, respectively. Chromatographic conditions were as described by Gresta *et al*. (2010). Using SPSS, mean values were compared by one-way ANOVA and principal component analysis (PCA) was applied for reducing the number of variables to a smaller number of the new derived variables (principal components, PCs) that adequately summarize the original information, i.e., the alkaloids composition of the studied lupin samples.

Major results and discussion

Nine compounds were identified comprising quinolizidine (lupinine, sparteine, angustifoline, α-isolupanine, lupanine, 11,12,-dehydrolupanine and 13α-hydroxylupanine), piperidine (smipine) and indole (gramine) alkaloids. Lupanine was the major alkaloid in samples of WL and NLL whereas sparteine was the most abundant compound in most of YL samples. These two tetracyclic quinolizidine alkaloids are ubiquitous in lupin species. Two PCs explained 73.23% of total data variability (Fig. 1). PC1 represented 47.44% of the variation and was associated with total alkaloids content, and with the compounds angustifoline, lupanine and 13α-hydroxylupanine, whereas PC2, responsible for 25.79% of the variation, was mainly represented by lupinine and sparteine. According to that, three groups of lupin samples could be clearly distinguished (Fig. 1).

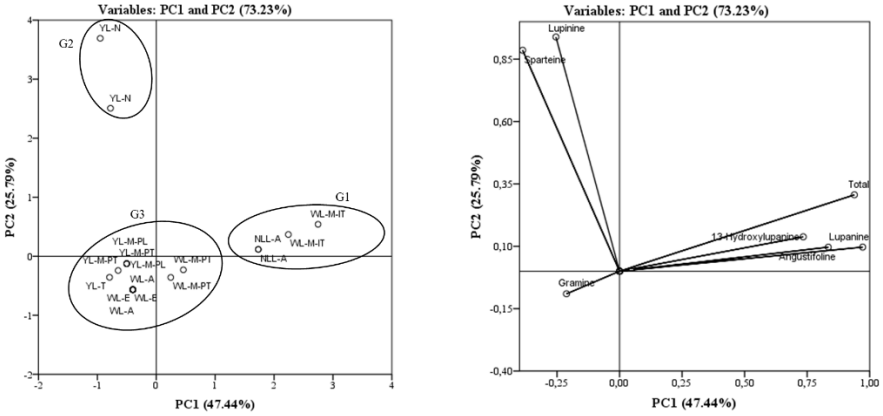

Fig. 1. Projection of lupin samples (variables: WL var. Estoril (WL-E); WL var. Amiga (WL-A); WL var. Multitalia-IT (WL-M-IT); WL var. Multitalia-PT (WL-M-PT); WL var. Lumen; YL ecotype Nacional (YL-N); YL var. Mister-PT (YL-M-PT); YL var. Mister-PL (YL-M-PL); YL var. Dukat (YL-D); YL var. Taper (YL-T); NLL var. Azuro (NLL-A); NLL var. Sonet (NLL-S)) and loadings by alkaloids and total alkaloids content into the plane composed by the principal components PC1 and PC2 containing 73.23% of the total variance.

Table 1 summarizes the total alkaloids content of each lupin sample, indicates which of them are suitable for human consumption and also reports their maximum level of inclusion in feedstuffs based on the maximum recommended concentration of individual alkaloids reported in the literature.

Table 1. Studied lupin samples: total alkaloids content and suitability as food and feed.

Lupin samples	Total alkaloids mg/100 g DM	Human consumption? if < 20 mg/100 g DM	Trout % of inclusion	Pigs
WL-E	19.8 (S)	Yes	-	38-56
WL-A	0.0 (S)	Yes	-	100
WL-M-IT	5169.1 (B)	No	-	< 1
WL-M-PT	1219.2 (B)	No	-	< 1
WL-L	31.5 (S)	No	-	27-40
YL-N	1030.7 (B)	No	~ 1	~ 1
YL-M-PT	26.7 (S)	No	38	19-41
YL-M-PL	70.6 (B)	No	14	7-16
YL-T	77.5 (B)	No	18	24-51
YL-D	12.4 (S)	Yes (yet, not from an edible lupin species)	81	41-89
NLL-A	2440.2 (B)	No	-	< 1
NLL-S	63.9 (B)	No	-	14-20

S, sweet (< 50 mg/100 g DM); B, bitter (> 50 mg/100 g DM)

Besides monogastrics, also ruminants (sheep, cattle) are large consumers of lupin seeds as protein sources. Their biggest advantage regarding dietary alkaloids is that, apparently, prolonged exposure of alkaloids to rumen microorganisms increase their tolerance to such metabolites and may suppress alkaloids deleterious effects (Aguiar and Wink, 2005). Nonetheless, under penalty of affecting feed intake, bitter varieties found in the present work, and especially those containing very high levels of alkaloids (> 1000 mg/100 g DM) should be debittered to ensure a safer consumption and to allow increasing lupins dietary levels in monogastrics and ruminants.

Conclusions

Sweet WL and YL varieties appear as good options to include in food and/or feedstuffs as high intakes or inclusion levels appear to be possible. For the other lupin seeds, a debittering process is recommended before consumption.

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ALKALOIDS IN THE VALORIZATION OF EUROPEAN *LUPINUS* SPP. SEEDS CROP

Sara C. Q. Magalhães[1]*, Fátima Fernandes[2], Ana R. J. Cabrita[1], António J. M. Fonseca[1], Patrícia Valentão[2], Paula B. Andrade[2]

[1] REQUIMTE/LAQV, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal.
[2] REQUIMTE/LAQV, Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, R. Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal.
E-mail: pandrade@ff.up.pt

Introduction

Lupins (*Lupinus* spp.) are low cost and non-genetic modified legume seeds that provide 30-40% dietary protein, ca. 28% fiber, healthy fatty acids, vitamins and minerals Sbihi, *et al.*, 2013. Besides, lupins also contain several phytochemicals that result from the plant secondary metabolism, alkaloids being major compounds. Their levels in the seed must be as low as possible to ensure a safe consumption of lupins Lucas, *et al.*, 2015. Indeed, in feedstuffs, lupins bitter taste, highly related to the seed alkaloids content, may decrease diet palatability, affecting feed intake and body weight gain; teratogenic alkaloids are of major concern for livestock due to death losses and to crooked calf disease in pregnant range cows Pilegaard and Gry, 2009. Although alkaloids may be toxic when ingested at high concentrations, several biological properties were already described for rich-alkaloid lupin extracts, such as antimutagenic, antibacterial, antifungal and anticancer Khan, *et al.*, 2015. As far as we are aware, the anti-inflammatory and antioxidant potential of these lupins secondary compounds has not been studied yet. The present work aimed at determining, in a cell-free system, the anti-inflammatory and antioxidant potential of rich-alkaloids extracts from seeds of European *Lupinus* species, at concentrations considered non-toxic when consumed, by evaluating the 5-lipoxygenase (LOX) inhibitory capacity and the nitric oxide radical ($^{\bullet}$NO) scavenging activity, respectively. As the 68th United Nations General Assembly declared 2016 as the International Year of Pulses United Nations, 2014, we consider of interest the study of a major group of phytochemicals in lupins also from a pharmacological perspective.

Material and methods

Eight varieties (included in the European Plant Variety Database PVD, 2015) and one Portuguese ecotype of lupins, corresponding to mature raw seeds of 3 white lupins (*L. albus*), 2 narrow-leafed lupins (*L. angustifolius*) and 3 yellow lupins (*L. luteus*), were analyzed (Table 1). Seeds were dried (65 ºC, 24 h) and grounded (1 mm). Alkaloids were extracted as according to Muzquiz, *et al.*, 1994 and Gresta, *et al.*, 2010, with slight modifications. The inhibitory effect on LOX and the antiradical activity of the extracts were assessed according to Pereira, *et al.*, 2015 and Vrchovska, *et al.*, 2007, respectively. In both assays, three experiments were performed in triplicate.

Table 1. IC_{25} (mg/mL) values for LOX inhibition by white (WL), yellow (YL) and narrow-leafed lupins (NLL) rich-alkaloid extracts.

Lupin varieties	Origin	Total alkaloids content (g/kg DM)	IC_{25} for LOX inhibition
WL Estoril	Portugal	0.19	0.136
Wl Multitalia-IT	Italy	51.69	0.525
Wl Multitalia-PT	Portugal	12.19	0.229
YL Nacional	Portugal	10.31	0.766
YL Taper	Poland	0.78	0.104
YL Dukat	Poland	0.12	0.341
NLL Azuro	Portugal	24.40	0.416
NLL Sonet	Poland	0.64	> 0.354

Results and discussion

The rich-alkaloid lupin extracts exhibited a concentration-dependent LOX inhibitory capacity (Figure 1). According to the effect observed (IC_{25}), Taper and Nacional were the most and the least potent varieties, respectively (Table 1). For Sonet, 18% of inhibition was noticed for the maximum concentration tested (0.354 mg of dried extract/mL). Pure compounds also inhibited LOX in a concentration-dependent manner, gramine displaying the strongest effect (data not shown). Due to low solubility in the phosphate buffer used in the assay, the highest concentration tested for lupanine, sparteine and angustifoline was 0.077 mg/mL, which corresponded to 13, 18 and 23% inhibition, respectively. Both lupin extracts and pure standards revealed lower inhibitory capacity than quercetin (IC_{25}=0.00051), the positive control used.

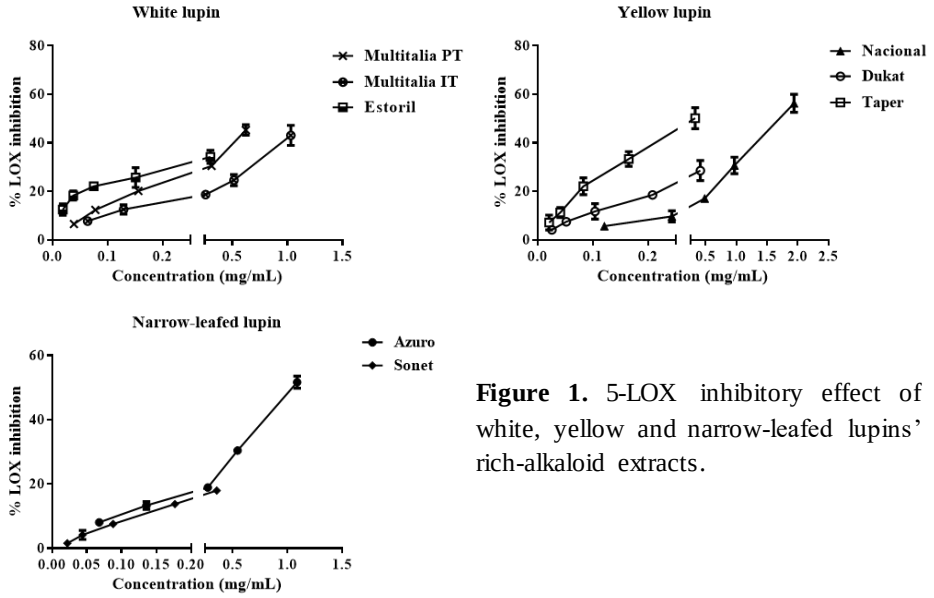

Figure 1. 5-LOX inhibitory effect of white, yellow and narrow-leafed lupins' rich-alkaloid extracts.

The rich-alkaloids extracts studied herein revealed a moderate LOX-inhibitory potential. There was not a direct relation between extracts activity and its total alkaloid content (Table 1) but these compounds contribute to some extent for the extracts activity; indeed, LOX inhibitory activity of Taper may be greatly attributed to gramine's activity. The results obtained suggest that besides the phenolic compounds previously reported Czubinski, *et al.*, 2012, alkaloids can play a role in LOX inhibition in lupin seeds.

All the extracts and pure compounds displayed weak activity against •NO, Azuro displaying the best scavenging activity (20% at the highest concentration. Gramine was able to be scavenge •NO up to 34% at the maximum concentration (1 mg gramine/mL). Lupanine (0.238 mg/mL) presented ca. 11% of activity, whereas spartein and angustifoline revealed no activity.

Conclusion

The studied rich-alkaloid lupin extracts showed moderate LOX inhibitory activity, explained, at least partially, by their alkaloid composition, but were weak •NO scavengers.

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