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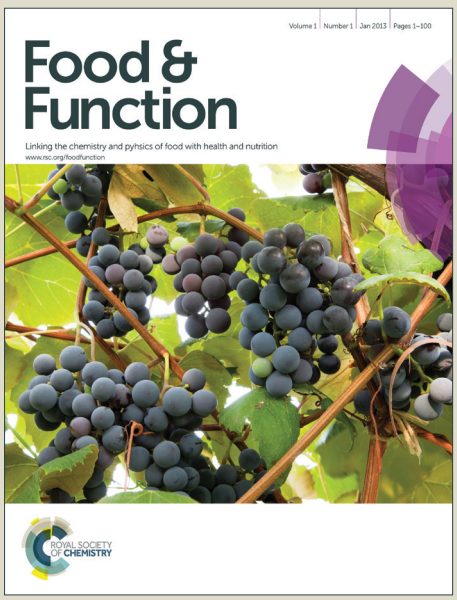

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Valle Agricola lentil, an unknown lentil (*Lens culinaris* Medik.) seed from Southern Italy as a novel antioxidant and prebiotic source

Nicola Landi [a, 1], Severina Pacifico [a, 1], Simona Piccolella [a], Antonella M. A. Di Giuseppe [a], Maria C. Mezzacapo [a], Sara Ragucci [a], Federica Iannuzzi [a], Armando Zarrelli [c], Antimo Di Maro [a, *]

[a]*Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, Second University of Naples, Via Vivaldi 43, 81100-Caserta, Italy*

[b]*Department of Organic Chemistry and Biochemistry, University of Naples 'Federico II', Via Cintia 4, 80126-Napoli, Italy*

*, E-mail: antimo.dimaro@unina2.it (ADM); *Tel*: +39 0823 274535, *Fax*: +39 0823 274571.

[1] These authors equally contributed to this paper

Abstract

In order to promote 'Valle Agricola' lentil, an autochthonous lentil of the Campania Region, a thorough investigation on its biochemical and nutritional properties has been carried out. Macronutrient content (proteins, carbohydrates and lipids), free and total amino acids, and unsaturated fatty acids, were determined. The antioxidant capability of raw 'Valle Agricola' lentils, as well as of boiled ones, was estimated in terms of their total phenol content (TPC), ORAC value, and free radical scavenging capacities using DPPH and ABTS assays. Data obtained evidenced that boiling process slightly decreased Valle Agricola lentils antioxidant power. Furthermore, when trypsin and chymotrypsin inhibitory activities were measured, an high decrease of the levels of anti-nutritional factors, were estimated. In order to have a phytochemical overview of this autochthonous lentil seed, LC-ESI-MS/MS analyses was applied on raw and boiled lentil extracts. Flavonols glycosides and free flavanols, as well as typical seed prebiotic saccharides, were the most representative constituents.

Keywords: Amino acids, Anti-nutritional factors, Antioxidant activity, *Lens culinaris* Medik, Nutritional value

1. Introduction

Exponential growth of the human population has led to serious environmental changes with progressive alteration of the natural environment and biodiversity loss. The development of chemical-industrial agriculture, and the tendency to uniform farming on productive and rewarding species have condemned the provision favouring the subsequent extinction of all landraces not producing adequate income [1]. Mediterranean area seems to be only partially affected. In fact, it still represents one of the largest sources of wild plant cultivars, many of which are locally cultivated and consumed as traditional food [2]. In different Italian regions, wild plants are expression of their cultures and are traditionally used for the preparation of "homemade" products, essentially destined to the local market [3-6]. In order to preserve the precious genetic variability of Italian local cultivars, their biochemical and nutritional valorisation has to be pursued. The knowledge of nutritional value of local plant species, and of the beneficial effects on human health arising from their consumption, could be a valid approach to support their cultivation and marketing [7].

In particular, it is interesting preserve the production of local legume species as the lentils. Lentil (*Lens culinaris* Medik.) is a nutritious pulse food whose cultivars are characterized by seeds that differ for color (range from yellow to red-orange to green, brown and black), size (*microsperma* or *macrosperma*) and form [8]. Generally consumed boiled as soup or processed into flour [9], lentils are reported to contain a large amount of inexpensive and accessible high quality proteins and is a well-balanced source of essential amino acids, carbohydrates, fibre, minerals, and vitamins [10]. Furthermore, as they are poorly rich in fats, and contain bioactive natural antioxidants, they are recognized an ideal complement to cereals in vegetarian diet and a functional food with a positive healthy impact [11, 12].

Italy is broadly known for its production of some lentil cultivars which are mainly cultivated in marginal areas of Central and Southern Italy. Among them, 'Castelluccio di Norcia' (Protected Geographic Indication; EC Reg. n°. 1065/97) and 'Colfiorito' (Umbria Region), 'Onano' (Lazio), 'Fucino' (Abruzzo), 'Altamura' (Puglia); 'Mormanno' (Calabria), 'Pantelleria' and 'Villalta'

(Sicilia) are renowned at international level[13]. Indeed, several other lentil cultivars are grown in Italy. In Campania region, for instance, lentil cultivars as 'San Geraldo' (Avellino), 'Colliano' (Salerno) and 'Valle Agricola' (Caserta) are locally produced and consumed.

'Valle Agricola' lentil is so called as it is from Valle Agricola, a small town localized, near Caserta, in the piedmont part of the Matese Mountain chain. It is a local ecotype, belonging to microsperma varieties, characterized by medium dimensions and a rather dark colour, with a thin skin and a characteristic flavour. Produced in low quantities through family-run terraces cultivation, these high-quality lentils are stored in large weave canvas bags, and sold mainly on the local market.

In order to valorize 'Valle Agricola' lentil and to encourage its consume at local and international levels, an extensive nutritional and biochemical investigation aimed to define its macronutrient content has been carried out (**Fig. 1**). Antioxidant capability of raw and boiled 'Valle Agricola' lentil material was estimated in terms of total phenol content and ORAC value of extracts properly prepared. As lentils are known to contain some anti-nutritional factors which could limit their consumption, trypsin and chymotrypsin inhibitory activities were also performed. LC-MS-based metabolic profile analyses were also performed on extracts from raw and boiled lentil seeds.

2. Experimental

2.1. Chemicals and reagents

2,2'-azobis-(2-amidinopropane)-dihydrochloride (AAPH), *nor*-leucine (nor-Leu), fluorescein, Folin-Ciocalteau reagent, gallic acid, hydrogen peroxide, peroxidase from horseradish, salts and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) were purchased from Sigma-Aldrich (Milan, Italy). Solvents were from Sigma-Aldrich. Chemicals and solvents for the Kjeldahl method were from Carlo Erba Reagents (Milan, Italy), whereas those for automated amino acid analysis were provided from Biochrom (Cambridge, U.K.). Fatty acid methyl esters (Supelco™ 37 component FAME mix) were obtained from Supelco (Park-Bellefonte, PA, USA).

2.2. Plant material and sampling

Lens culinaris seeds were grown under typical soil, and illumination conditions in Valle Agricola, 71 km Northwest from Caserta, Italy (geographical coordinates: 41°25'33"60 N, 14°15'21"24 E). Lentil plants (~40 cm tall), manually and randomly harvested across the reference field on July 2013, were sun-dried for one week. Dried lentil seeds were powdered with the Cyclone Sample Mill Instrument (PBI International, Milan, Italy), until flour of homogeneous size was obtained. All measurements were performed by triplicate.

2.3. Ash and moisture content

Ash content and moisture level were determined according to the AOAC official method [14].

2.4. Macronutrient content.

Total protein content: Nitrogen concentration was obtained by the Kjeldahl method [14] and total protein content was estimated using a nitrogen factor of 6.25. Samples (~1.0 g each) were analysed

using a Mineral Six digester and an Auto Disteam semi-automatic distilling unit (VWR International PBI, Milan, Italy). *Total lipid content*: Aliquots (2.0 g) of frozen lentil flour sample were lyophilized using a FTS-System Flex-Dry™ instrument (SP Scientific, Stone Ridge, NY USA). Thus, the freeze-dried materials were extracted for 4 h through a Soxhlet apparatus using $CHCl_3$ as extracting solvent. The solutions obtained were dried by a rotary evaporator in order to provide the crude fat extracts. The lipid content was determined gravimetrically. *Total carbohydrate content*: Total carbohydrates were obtained by subtracting (moisture + crude protein + crude fat + ash + total polyphenol + free sugar) from 100[15].

2.5. Amino acids composition

For the analysis of free amino acid composition, three aliquots of ~200 mg of seed flour were precipitated with 80% cold ethanol (1.0 mL), in the presence of *nor*-Leu (50.0 nmol) as internal standard, homogenized with a teflon pestle and centrifuged at 14000 ×*g*, at 4 °C. The supernatants were lyophilized, treated with 3% sulfosalicylic acid (500 µL) to precipitate any protein fraction still present, and centrifuged again[16]. Thus, the supernatants obtained were analysed. For the analysis of total (free and protein) amino acids, ~10 mg of seed flour were hydrolysed with 0.5 mL of 6 N HCl containing 0.02% phenol and *nor*-Leu as internal standard at 110 °C for 20 h [4]. Following hydrolysis, HCl was removed under vacuum and samples were re-suspended in 0.5 mL of 0.2 M lithium citrate buffer (pH 2.2). Aliquots of hydrolysed and non-hydrolysed samples were directly analysed on a Biochrom 30 amino acid analyser (Biochrom, Cambridge, U.K.), equipped with a post-column ninhydrin derivatization system.

2.6. Gas chromatographic analysis of fatty acid methyl esters

The analysis of fatty acid methyl esters content were performed as previously reported [4]. Briefly, each $CHCl_3$ crude extract (1.0 mg; see "Macronutrient content" section) was dissolved in 2.0 mL of 2 N KOH in MeOH. The solution was stirred for 30 min at room temperature, added with heptanes

(0.8 mL), vortexed and then centrifuged, using a Beckman GS-15R centrifuge (Beckman Coulter Italia), for 10 min at 4200 ×*g*. Organic upper phase (1.0 µL) was analysed by GC (100 m × 0.25 mm i.d., 0.2 µm SP2380, fused silica capillary column; Supelco, Sigma-Aldrich). The fatty acid methyl esters were identified by comparing their retention times with those of the standard fatty acid methyl esters (Grain Fatty Acid Methyl Ester mix 47801, Supelco).

2.7. Free sugar (pentoses and hexoses) content

For the analysis of monosaccharide composition, three aliquots of about 50 mg of seed flour were precipitated with 80% cold ethanol (1.0 mL) homogenized with a teflon pestle and centrifuged at 14000 ×*g*, at 4 °C. Thus, the supernatants obtained were determined using a Bio-LC® (Dionex Corp., Sunnyvale, CA, USA) equipped with a CarboPac® PA10 column (2 × 250 mm, Dionex Milan, Italy) and a guard column Amino Trap™ (2 × 50 mm, Dionex). Protocols employed were as suggested by the manufacturer (Technical Note 40 by Dionex Corp.).

2.8. Evaluation of antioxidant capability of 'Valle Agricola' lentil flour

Total phenol content (TPC) and antioxidant capability of 'Valle Agricola' lentil flour were measured on methanol extracts, previously obtained through ultrasound-assisted maceration.

To this purpose three replicate lentil flour samples (2.0 g each) underwent ultrasound assisted extraction (Dr. Hielesher UP 200S, Germany) using pure methanol as extracting solvent (50 mL for each sample). Four sonication cycles were performed (30 min each) in order to achieve the maximum recovery of the lentil flour metabolic content. At the end of each sonication cycle, samples were centrifuged at 2,044×*g* for 10 min in a Beckman GS-15R centrifuge (Beckman Coulter, Milano, Italy) fitted with rotor S4180. Obtained supernatants were dried under vacuum by a rotary evaporator (Heidolph Hei-VAP Advantage, Germany) to yield crude extracts, which were stored at -20°C until use.

Furthermore, in order to evaluate the effect of boiling (the most used cooking method for lentils consume) on phenol content and antioxidant capacity, three replicate samples (2.0 g each) of raw material was first boiled in water (50 mL, each). After 30 min, the extracts were centrifuged at 4500 ×*g*. Cooking water (supernatant) was directly investigated for its TPC value and antioxidant power, whereas the pellet underwent an extraction with methanol, as described above. Folin-Ciocalteau, ABTS, DPPH, and ORAC assays were carried out performing three replicate measurements for three samples (n=3) of each extract. Recorded activities were compared to a blank. Results are the mean ± SD values. Student *t*-test was applied in order to determine statistical significance (significance level was set at $P < 0.05$).

2.9. Total phenol content determination

Total phenol amount of 'Valle Agricola' lentil flour was determined according to the Folin–Ciocalteau procedure on aliquots of extracts in methanol. The content of total phenols (TPC value) of the samples was expressed as mg gallic acid equivalents (GAE) per g of seed flour.

2.10. Determination of ABTS radical cation scavenging capacity

The determination of $ABTS^{\bullet+}$ solution scavenging capacity was estimated as previously reported [5]. The results were expressed in terms of TEAC values (μmol Trolox® Equivalents per g of extract).

2.11. Determination of DPPH radical scavenging capacity

The determination of $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) scavenging capability was estimated as previously reported[5]. The results were expressed in terms of TEAC values (μmol Trolox® Equivalents per g of extract).

182 **2.12. Oxygen radical absorbance capacity assay (ORAC)**

183 The antioxidant potential of 'Valle Agricola' lentil flour was measured by Oxygen Radical
184 Absorbance capacity (ORAC) assay. The analysis was performed using 96-well microplates in
185 which 25 μL of appropriately diluted methanolic extract from raw and cooked lentils, as well as of
186 extract from lentil boiling water, were mixed with fluorescein (FL) probe [150.0 μL (80 nM, final
187 concentration) in 7.5 mM Na-phosphate buffer, pH 7.0 (Na/P)] and preincubated for 10 minutes at
188 37 °C. Then, 2,2'-azobis-(2-amidinopropane)-dihydrochloride (AAPH) solution [25.0 μL (18.4
189 mM, final concentration] was rapidly added. In parallel with the test samples, a blank (25 μL Na/P,
190 150 μL FL and 25 μL AAPH) and solutions of the reference antioxidant Trolox® (6.25-50 μM, final
191 concentration levels) were properly prepared in Na/P with 150 μL FL and 25 μL AAPH. The
192 fluorescence decay (λex = 485 nm, λem = 525 nm) was recorded every minute for 75 min using a
193 Tecan SpectraFluor fluorescence and absorbance reader. Antioxidant curves (fluorescence *vs*. time)
194 were normalized to the curve of the blank. From the normalized curves, the area under the
195 fluorescence decay curve (AUC) was calculated. Linear regression equations between net AUC
196 ($AUC_{antioxidant}$ - AUC_{blank}) and antioxidant concentration were calculated. The antioxidant activity
197 (ORAC value) was calculated by using the Trolox® calibration curve. The ORAC values were
198 expressed as Trolox® equivalents (μmol per g).

199

200 **2.13. Determination of Relative Antioxidant Activity Index (RACI)**

201 As the units and the scale of the data from various chemical methods are different, the data in each
202 data set should be transformed into standard scores, a dimensionless quantity derived by subtracting
203 the mean from the raw data divided by the standard deviation, according to the equation standard
204 score = $(x - \mu)/\sigma$, where x represents the raw data, μ the mean, and σ the standard deviation. The
205 standard scores of a sample for different assays when averaged give a single unit less value termed
206 as RACI, which is a specific combination of data from different chemical methods with no unit
207 limitation and no variance among methods [17].

208

209 **2.14. Determination of trypsin and chymotrypsin inhibitory activities**

210 Three replicate samples of 'Valle Agricola' lentil flour (1.0 g each) were extracted overnight under
211 magnetic stirring at 4 °C in 80 mM Tris•Cl, pH 7.8, containing 0.1 M $CaCl_2$ (1:4; w/v). The
212 reaction mixtures were centrifuged at 24000 ×*g* (Centrifuge Avant J-25, Beckman Coulter, CA,
213 USA) at 4 °C for 60 min. The supernatants were first filtered on Miracloth (pore size: 22-25 μm)
214 and then freeze-dried.

215 The protein concentration was determined with the Bio-Rad Protein Assay kit following the
216 manufacturer's instructions and using bovine serum albumin (BSA) as standard.

217 Trypsin and anti-trypsin activities, as well as chymotrypsin and anti-chymotrypsin activities, were
218 determined following a procedure already described [18], using TAME (*p*-toluenesulfonyl-L-arginine
219 methyl ester) and BTEE (N-benzoyl-L-Tyrosine ethyl ester) as substrates, respectively. One
220 inhibitory unit is defined as the amount of inhibitor that, under the assay conditions, yields a 30%
221 decrease of the assayed enzymatic activity. Increasing concentration of lentil protein extract was
222 added to a fixed concentration of enzyme that in our condition corresponding to 0.05
223 absorbance/min monitoring for 5 min the reaction. Residual enzyme activities are expressed as
224 percentage of the control samples (enzyme activity in the absence of protein extract extracted). The
225 IC_{50} values on trypsin and α-chymotrypsin activities (i.e. the half maximal inhibitory concentration)
226 of the lentil protein extract were calculated as previously reported[18].

227

228 **2.15. LC and LC-MS/MS analyses**

229 In order to provide a preliminary phytochemical overview of 'Valle Agricola' lentil seeds, LC-ESI-
230 MS/MS analysis were applied on raw and boiled lentil methanolic extracts (see section 2.8).

231 For the LC/MS analysis, a Waters 2695 LC system, equipped with a Waters 2487 dual λ absorbance
232 detector, was coupled to a Quattro Micro (Micromass, Manchester, UK) triple-quadrupole
233 instrument.

A Phenomenex® Gemini C18 column (3.0 µm particle size, 150 mm × 2.00 mm) together with a pre-column was used for chromatographic separation. The mobile phase consisted of A: 0.1% formic acid in water and B: acetonitrile. Starting with 95% A, a linear gradient was followed to 85% A in 10 min, then decreasing to 75% A at 30 min, and to 20% A at 40 min, then returning to starting conditions and allowing to re-equilibrate for 5 min. The total analysis time was 46 min, the flow rate was 0.3 ml/min, the detection wavelength was set at 280 nm and the injection volume was 5.0 µL. Electrospray ionization (ESI) mass spectra were acquired in the negative ion mode. Nitrogen was used as the nebulization and desolvation gas at flow rates of 50 and 500 l/h, respectively. The source and desolvation temperatures were set at 120 and 500°C. The applied potentials on the electrospray capillary and on the cone ranged from 2.5 to 3.5 kV and from 15 to 55 V, respectively, and were optimized for each molecule. CAD mass spectra were obtained by introducing argon as a collision gas into the radiofrequency (Rf)-only quadrupole. The Ar pressure was kept low (~3.0×10^{-3} mbar) to minimize multiple collisions. Collision energy was varied from 10 to 45 eV and optimized for each molecule. Data acquisition and processing were carried out using the software MassLynx™ version 4.0 supplied with the instrument. Full scan mass spectra were acquired from *m/z* 50 to 1500 in MS and from *m/z* 20 to the *m/z* value of the precursor ion in MS/MS experiments, with a scan time and an interscan time of 0.2 and 0.1 s, respectively.

2.16. Statistical analysis

Analyses were repeated three times for each sample; mean and standard deviation (SD) of experimental values are reported. Data analysis was carried out with Excel Office 2010 (Microsoft Corporation, Redmond, WA, USA). The IC_{50} values were calculated on the basis of inhibition curves by plotting the residual enzyme activities versus different concentrations of protein extract by fitting data with a non-linear regression analysis on a semi-logarithm scale by using the GraphPad Prism 5 software (GraphPad software Inc., California, USA). All measurements were performed in triplicate with standard deviations always below 5% for each experiment

260 ## 3. Results and discussion

261

262 ### 3.1. Nutritional values

263 Nutritional values of *L. culinaris* seeds from 'Valle Agricola' are reported in **Table 1** and compared
264 with values reported by the Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione (INRAN)
265 for Italian raw lentil (hereafter INRAN lentil). The protein content of 'Valle Agricola' lentil (26.3
266 g/100 g) was higher than INRAN lentil (22.7 g/100 g) while lipids (2.5 g/100 g) was 2.5-fold than
267 INRAN lentil (1.0 g/100 g). Our results showed that protein content of 'Valle Agricola' lentil are
268 similar to Colfiorito (27.1 g/100 g), Colliano (26.0 g/100 g) and Altamira (25.5 g/100 g) cultivars [19].
269 In addition, the lipids content was higher than that the above cited cultivars. However, it was in
270 accordance with the previously reported range 0.3-3.5 g/100 g [20] confirming that lentil are a low
271 source of lipids. The content of carbohydrates is 56.72% (range 43.3-74.9 g/100 g published for
272 other lentils [20]), slightly higher than that reported for INRAN lentil (about 1.11-fold). The amounts
273 of saccharose, glucose and fructose were 81.6 mg/100 g, 11.4 mg/100 g and 1.0 mg/100 g,
274 respectively, with a glucose/fructose ratio of 11.63. The low rate of free sucrose, as well as of its
275 hexose components, allowed us to hypothesize that the carbohydrate amount consisted mainly in
276 resistant starch (RS), dietary fiber (DF) and oligosaccharides, which are reported as the main pulse
277 carbohydrate fractions[21]. Peculiarly, lentils are known to contain galactooligosaccharide (GOS),
278 with prebiotic effects, which, primarily via bifidogenesis, include increased calcium absorption and
279 pathogen reduction[22].

280

281 ### 3.2. Amino acid content

282 The content of free and total (free plus protein) amino acids is reported in **Table 2**. Comparing the
283 total amino acid content of 'Valle Agricola' lentil with INRAN one, both qualitative and

quantitative differences were found. The total amino acid content for 100 g product of 'Valle Agricola' was about 1.17-fold that that of INRAN lentil (24.8 and 21.2 g, respectively).

Glx (glutamic acid + glutamine) was the most abundant among total amino acids (about 18% for both 'Valle Agricola' lentil and INRAN lentil). Among the other amino acids in 'Valle Agricola' lentil, abundant, in decreasing order, were Asx (aspartic acid + asparagine) (12.5%), arginine (8.17%), leucine (8.05%) and serine (7.12%), overall accounting for about 35% of the total. In addition, the amount of essential amino acids [His, Ile, Leu, Lys, Met, Phe, Thr, Val; Trp (tryptophan is not included as it was not determined in the total hydrolysed samples – see Table 2)] in 'Valle Agricola' lentil was 8.9 g/100 g *vs*. 8.0 g/100 g in INRAN lentil. The amount of both methionine and cysteine (as cysteic acids) in 'Valle Agricola' lentil did not exceed 0.45 g/100 g of product represented 1.81% of total amino acids confirming the low content of sulphur containing amino acids as reported for other legumes.

Considering free amino acid, the total amount in 'Valle Agricola' lentil was 147.6 mg/100 g. The analysis evidenced the presence of several non-protein amino acids. Asparagine was by far the most abundant among free amino acids (about 30%). Furthermore, α-aminoadipic acid (15.8 mg/100g), glutamic acid (8.9 mg/100g) and serine (8.1 mg/100g) were among the free amino acids the most abundant in 'Valle Agricola' lentil, whereas the amount of each of the other non-protein amino acids (carnitine, γ-amino-*n*-butyric acid, hydroxylysine, hydroxyproline, 1-methylhistidine, *o*-phospho-serine, sarcosine and taurine) did not exceed 23 mg/100 g of product (13.6% total content of free amino acid). Furthermore, the amount of free essential amino acids, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val, in 'Valle Agricola' lentil was 12.6 mg/100 g *vs*. 8.9 g/100 g (not significant as contribution in a human diet).

3.3. Fatty acid composition analysis

GC-MS analysis showed that 'Valle Agricola' lentils have relatively low fat. In particular, the fatty acid composition was more rich in unsaturated acid content (301 g/100 g; 73.6%) than in saturated

one (108 g/100 g; 26.4%; **Table 3**). The most abundant unsaturated acids were oleic (C18:1 c) and eicosenoic (C20:1) acids, which represented 34.3% and 17.4% respectively, of total fatty acids, whereas no traces of linolenic acid were detected. Indeed, the presence of α-linolenic acid was reported to be related to lentil germination and it achieved high value in lentil sprout[23]. Erucic acid, a monosaturated ω-9 fatty acid, was also identified in appreciable level (34.5 mg/100 g). The presence of this latter fatty acid, was previously reported as minor fatty acid constituent of red lentil seeds[24]. Dietary erucic acid (13-*cis*-docosenoic acid) is used in the management of the adrenoleukodystrophy as a glyceryl trierucate from which is commonly called as Lorenzo's oil [25].

3.4. Anti-proteinase inhibitor activity

Lentils are a significant dietary source of protease inhibitors such as trypsin- and chymotrypsin inhibitors that are present at higher concentrations in lentils seeds which certainly interfere with protein digestion[26]. In this framework, the anti-trypsin as well as anti-chymotrypsin assays were carried out on protein extract (~22.5 mg/ g of seeds) obtained from 'Valle Agricola' lentil flour and the inhibitory activity was reported as IC_{50} value. The anti-chymotrypsin activities of 'Valle Agricola' lentil (IC_{50} 4.6) were 2-fold higher than the anti-trypsin activities (IC_{50} 8.9), while not protease activities were detected in same experimental conditions. On the other hand, to confirm the decreasing effect of boiling process on trypsin inhibitor content [27], aliquots of 'Valle Agricola' lentil seeds were boiled (30 min) and the anti-protease activity re-assayed on protein extract (~2.0 mg/ g of boiled seeds). Overall, these results indicated that the anti-chymotrypsin as well as anti-trypsin activities were 10.45-fold (IC_{50} 48.3) and 6.12-fold (IC_{50} 54.4) lower than the detected in raw lentil seeds, respectively. Therefore, boiling process, as previously reported for other legumes, reduces the antiprotease activities of 'Valle Agricola' lentil seeds and any their adverse effects in humans[28].

3.5. Total phenol content and antioxidant capacity of raw and boiled 'Valle Agricola' lentils

In order to determine the total polyphenol content (TPC) of 'Valle Agricola' lentil seeds, they were extracted through ultrasound assisted maceration using methanol as extractant. The calculated TPC value was compared to that obtained from an equal amount of lentils, previously boiled in water for 30 min, as in the common culinary tradition. To this purpose, lentils, once cooked, were filtered and extracted by ultrasound-assisted maceration in methanol. The polyphenol content of boiling water was also measured. TPC value of raw lentils, 23.1±0.4 mg GAE per g, was slightly higher than that of cooked lentils (20.3±0.5 mg GAE per g), whereas a poor amount of phenol substances was released in boiling water samples (2.8±0.2 mg GAE per g). Thus, the effect of conventional boiling treatment seemed to influence only weakly the 'Valle Agricola' lentil polyphenol content. This finding was in line with Aguilera and co-authors[29], who evidenced that thermal processing of the 'Pardina' lentil flour apparently did not affect its total phenol content and antioxidant activity. Indeed, performing HPLC-PAC and HPLC-MS (ESI) analysis on raw and processed materials, they found that catechins and procyanidins, flavonols, flavones, and flavanones decreased, while hydroxybenzoic compounds exhibited an important increase. On the contrary, Xu and coauthors[30] reported that different four thermal processing methods (conventional boiling, conventional steaming, pressure boiling, and pressure steaming) caused significant reduction in total phenolic content, antioxidant capacities, and antiproliferation properties of lentils and other commonly consumed cool-season food legumes. Han and Baik[9] reported that TPC of lentils was higher than that of chickpeas, peas and soybeans; it was only ~12 mg per g for *cv*. 'Pardina' and 'Crimson'. According to Kalogeropoulos and coauthours[31], who reported that lentils score the highest TPC values among 14 different types and varieties of pulses, 'Valle Agricola' raw lentil seeds had a TPC value of about 23.1 mg GAE per g of material.

The evaluation of antiradical properties of the three extracts, carried out performing DPPH, ABTS methods, as well as ORAC one, revealed that boiling treatment slightly decreased 'Valle Agricola' lentils free radical scavenging efficacy (**Fig. 2**). Data obtained from ABTS, DPPH, ORAC, and Folin-Ciocalteau reagent (FCR) tests were used to calculate RACI (*Relative*

362 *Antioxidant Capacity Index*) value for each extract. The data obtained by applying ABTS method
363 positively correlated to those from DPPH method (0.977), ORAC assay (R = 0.997), and to TPC
364 value (0.907). Similarly, a positive correlation (**Table 4A**) was observed between the DPPH data
365 and those of ORAC (R = 0.991) and TPC data, R = 0.976). RACI calculation represents the average
366 of the standard scores obtained from the raw data for the various methods (**Table 4B**). The results
367 emphasized that the raw lentils were able to display an antioxidant capability (RACI 0.716) slightly
368 stronger than cooked lentils (RACI 0.617), whereas cooking water did not show antioxidant effects
369 (RACI -1.151). As polyphenols are considered the main secondary metabolites affecting the
370 antioxidant power of plant extracts, data obtained was explained assuming the great amount of these
371 compounds in the vacuoles. In fact, although flavonoid glycosides are drastically reduced by
372 thermal processing, this latter could trigger the release of more bound phenolic acids during
373 breakdown of cellular constituents and membranes[12], and promote the conversion of glycosides
374 constituents, mainly present at lentil seed coat level, in their relative free aglycones. The high
375 carbohydrate content in "Valle Agricola" lentil seeds could be also involved because these
376 compounds, commonly referred as dietary fiber constituents, are able to act as carrier of dietary
377 antioxidants[32]. The previously reported increase in metal chelating activity of cooked lentils seeds[33]
378 was, for instance, attributed to the formation of Maillard browning pigments by interaction of
379 carbohydrates (especially reducing sugars) and proteins during cooking. Furthermore, Agil *et al.*
380 (2013) found that the polysaccharide lentil fraction exhibited a strong antioxidant potential[34].
381
382 **3.6. LC-MS-based metabolite profile of 'Valle Agricola' lentil seed extracts**
383 To identify the main constituents in 'Valle Agricola' lentil seeds, LC-ESI-MS/MS analyses of raw
384 and boiled extracts, prepared by ultrasound assisted maceration, were carried out. The identified
385 metabolites are listed in **Table 5**. Compounds **1-5** were tentatively identified as oligosaccharides. In
386 particular, compound **1** showed the $[M-H]^-$ ion at *m/z* 827 and MS^2 fragments ions at *m/z* 665, 503,
387 341 and 179, which were formed by cleavage of the glycosidic bond with the loss of one, two, three

and four hexose moieties (-162 Da), respectively. Compound **2**, characterized by a $[M-H]^-$ ion at *m/z* 517, was hypothesized to be a dihexosyl cyclitol, likely ciceritol [35]. Its MS^2 spectrum provided the fragment ion at *m/z* 193, due to the neutral loss of two hexose residues (-324 Da). This compound, together with other galactosides of myo-inositol isomers, are broadly accumulated in legume species seeds[36]. The accumulation of galatosyl cyclitols in seeds starts at the stage of pod filling and increases during seed maturation and desiccation steps[37]. Compounds **3**-**5** were putatively identified as 6-carbon tetrasaccharide, trisaccharide and disaccharide, respectively. In particular they could be α-Galactosides of the raffinose family (RFO), which were found as the most abundant oligosaccharides in lentil seeds. The raffinose series oligosaccharides contents in legumes are known to increase with maturation progress[38] and they have been also related to the ability of legumes to survive dry periods[39]. All these compounds are referred as prebiotic carbohydrates that are important components of healthy diets, supporting healthful hindgut microflora. Prebiotic concentrations in lentils appear to be related to genetic and environmental factors[40]. When boiling process, it was found that cooking water was particularly enriched in these compounds, on the basis of their huge solubility in water [41].

Flavonol glycosides were abundantly present. Compounds **6**, **9**, **11**, **13** and **14** were identified as kaempferol glycosides. In particular, the MS^n spectra of compound **6**, which showed its $[M-H]^-$ ion at *m/z* 901, were in accordance with the presence of a kaempferol tetraglycoside. This compound, together with both the detected kaempferol triglycoside isomers (**9** and **11**) were previously identified as a component of lentil seeds and aerial parts[42, 43]. The MS^2 spectrum of the deprotonated metabolites **9** and **11** at *m/z* 755 showed fragment ions at *m/z* 593 $[M-H-162]^-$, 285 (kaempferol aglycone) and 284 (radical kaempferol)[44]. Compounds **13** and **14** were tentatively identified as hexosyl and pentosyl kaempferol, respectively. The collision of the $[M-H]^-$ ion (*m/z* 447 for **13** and 417 for **14**) provided a radical aglycone at *m/z* 284, as well as the $[aglycone-H]^-$ ion at *m/z* 285, attributable to kaempferol. Metabolites **7** and **8** were identified as quercetin derivatives. The MS/MS analyses allowed us to detect neutral losses of 162 and 176 Da, which were characteristic

414 of the presence of a residue of hexose, and hexuronic acid, respectively. The ion at *m/z* 301 in both
415 the cases was found to be the base peak. In raw lentil extract, pure quercetin (**17**) was also identified
416 on the basis of the comparison of its retention time and mass spectra with those of a standard pure
417 compound. 'Valle Agricola' flavanol constituents were represented by catechin (**14**) and epicatechin
418 (**15**), as well as by their derivatives characterized by a galloyl B-ring (**21** and **22**). Compounds **10**
419 and **12** likely corresponded to procyanidin dimers. In fact, both the metabolites showed a
420 deprotonated molecular ion at *m/z* 577, which after collision-activated fragmentation provided the
421 fragment ion at *m/z* 289, which, in turn, could correspond to either catechin or epicatechin[45]. The
422 MS/MS spectra of compound **18** gave the fragment ion at *m/z* 179, suggesting the presence of a
423 caffeoyl moiety in this compound. The neutral loss of 162 Da allowed us to tentatively identify it as
424 a caffeoyl hexose. Compound **20** likely corresponded to ferulic acid, whereas compound **19** ([M-
425 H]$^-$ at *m/z* 339) could be its deoxyhexosyl derivative.
426
427

Conclusions and perspectives

'Valle Agricola' lentil was particularly rich in proteins and low in lipids. These nutritional features make it a good food for diets low in energy content. All protein amino acids were identified, and the quali-quantitative analysis of free amino acids was reported for the first time. Raw lentil seeds contained an appreciable amount of polyphenol compounds and were able to exert a significant antioxidant capability. When boiling process was applied, 'Valle Agricola' lentil antioxidant power was retained suggesting that the regular consumption of this product could maximize the dietary intake of health beneficial compounds with protective or disease preventive properties. Indeed, thermal process applied increased the wholesomeness of the 'Valle Agricola' lentils as a strong decrease of trypsin and trypsin-chymotrypsin inhibitors, common legumes anti-nutrients, was detected. Overall, our data showed that 'Valle Agricola' microsperma lentils are a good source of nutrients and bioactive compounds able to provide health benefits.

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Conflict of interest

The authors have no conflicts of interest

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534 **Figure captions**

535

536 **Fig. 1.** Schematic workflow of the experimental strategy for the biochemical and nutritional
537 characterization of Valle Agricola lentils. SE = Soxhlet Extraction; LLE = Liquid Liquid Extraction

538

539 **Fig. 2.** Antioxidant Capacity in both raw and cooked lentils and cooking water expressed as μmol
540 Trolox Equivalents (TE) per g of each sample ± SD from A) ABTS method data; B) DPPH method
541 data; C) ORAC method data.

Table 1. A; nutritional value of Valle Agricola lentil seeds. B, Values are means (±SD) of triplicate analyses (n = 3) and are expressed on weight basis.

	***L. culinaris* Valle Agricola (g/100g)**	***L. culinaris* INRAN (g/100g)**
Ash	3.5±0.1	n.r.
Moisture	8.6±0.2	11.2
Proteins	26.3±0.5	22.7
Lipids	2.5 ±0.2	1.0
Carbohydrates	56.7	51.1

n.r., not reported

Table 2. Total and free amino acid composition of Valle Agricola lentil seeds. Protein amino acids are reported in bold. Values are means (±SD) of triplicate analyses (n = 3) and are expressed on weight basis.

	Valle Agricola lentil		*Lentil INRAN*
Amino acid [a]	Total amino acids [b] (g/100g)	Free amino acid content [c] mg/100g	Total amino acids (g/100g)
essential amino acids			
His	0.25±0.01	1.3±0.2	0.56
Ile	1.1±0.1	0.90±0.04	1.04
Leu	2.0±0.1	1.0±0.02	1.35
Lys	1.9±0.1	0.26±0.02	1.60
Met	0.21±0.01	0.05±0.02	0.19
Phe	1.3±0.1	2.2±0.1	1.19
Thr	1.1±0.05	4.1±0.1	0.89
Trp	n.d.	1.1±0.5	0.02
Val	1.0±0.05	1.6±0.2	1.19
non-essential amino acids			
AAAA	-	15.8±0.2	-
Ala	1.3±0.1	6.8±0.1	0.99
Arg	2.0±0.1	8.8±0.2	1.74
Asn	-	50.8±0.7	-
Asx	3.0±0.5	-	2.67
Asp	-	2.4±0.1	-
Car	-	4.3±0.3	-
Cys	-	-	0.21
Cysteic acid	0.24±0.04	-	-
GABA	-	0.43±0.02	-
Gln	-	1.2±0.1	-
Glx	4.5±0.3	-	3.87
Glu	-	8.9±0.2	-
Gly	1.3±0.1	1.5±0.1	0.94
Hylys	-	0.84±0.09	-
Hypro	-	4.8±0.3	-
1-mhis	-	1.9±0.1	-
Phser	-	5.9±0.2	-
Pro	1.2±0.2	4.1±0.4	0.94
Sarc	-	5.0±0.5	-
Ser	1.8±0.1	8.1±0.1	1.26
Taur	-	1.7±0.1	-
Tyr	0.71±0.03	1.2±0.1	0.70
Total (g)	24.85	0.148	21.23

[a] free and protein amino acids. Three letter code has been used: 1-mhis, L-1-methylhistidine; AAAA, L-α-aminoadipic acid; Ala, L-alanine; Asn, L-asparagine; Asx, L-asparagine + L-aspartic acid; Arg, L-arginine; Asp, L-aspartic acid; Carn, carnitine; Cys, L-half cystine; GABA, γ-amino-n-butyric acid; Gln, L-glutamine; Glu, L-glutamic acid; Glx, L-glutamine + L-glutamic acid; Gly, glycine; Hylys, L-Hydroxylysine, Hypro, L-Hydroxyproline; His, L-Histidine; Ile, L-isoleucine; Leu, L-leucine; Lys, L-lysine; Met, L-methionine; Phe, L- phenylalanine; Phser, o-phospho-L-serine; Pro, L-proline; Sarc, sarcosine; Ser, L-serine; Taur, taurine;, Thr, L-threonine; Trp, L-tryptophan; Tyr, L-tyrosine; Val, L-valine; n.d., not determined.

[b] free plus protein-derived amino acids (see text).

[c] free amino acids content for lentil INRAN and other cultivars is not previously published.

Table 3. Fatty acid constituents of Valle Agricola and INRAN lentil seeds. Values are means (±SD) of triplicate analyses (n = 3) and are expressed on weight basis.

		Valla Agricola lentils	*Lentil INRAN*
Fatty acid		Fatty acid content (mg/100g)	Fatty acid content (mg/100g)
Saturated			
palmitic	C16:0	*traces*	120.0
heptadecanoic	C17:0	37.5±2.1	-
stearic	C18:0	*traces*	10
docosanoic acid	C22:0	70.5±7.8	-
Unsaturated			
elaidic	C18:1 t	29.5±2.1	-
oleic	C18:1 c	140±3	170
eicosenoic	C20:1	71±3	-
erucic	C22:1	35±2	-
linoleic	C18:2	25.5±7.8	360
α-linolenic	C18:3	-	100
Total fatty acids		409	760

Table 4. A, correlation coefficients between the different antioxidant methods applied; B, standard scores of the antioxidant capability of each extract for each applied method and relative RACI values.

A

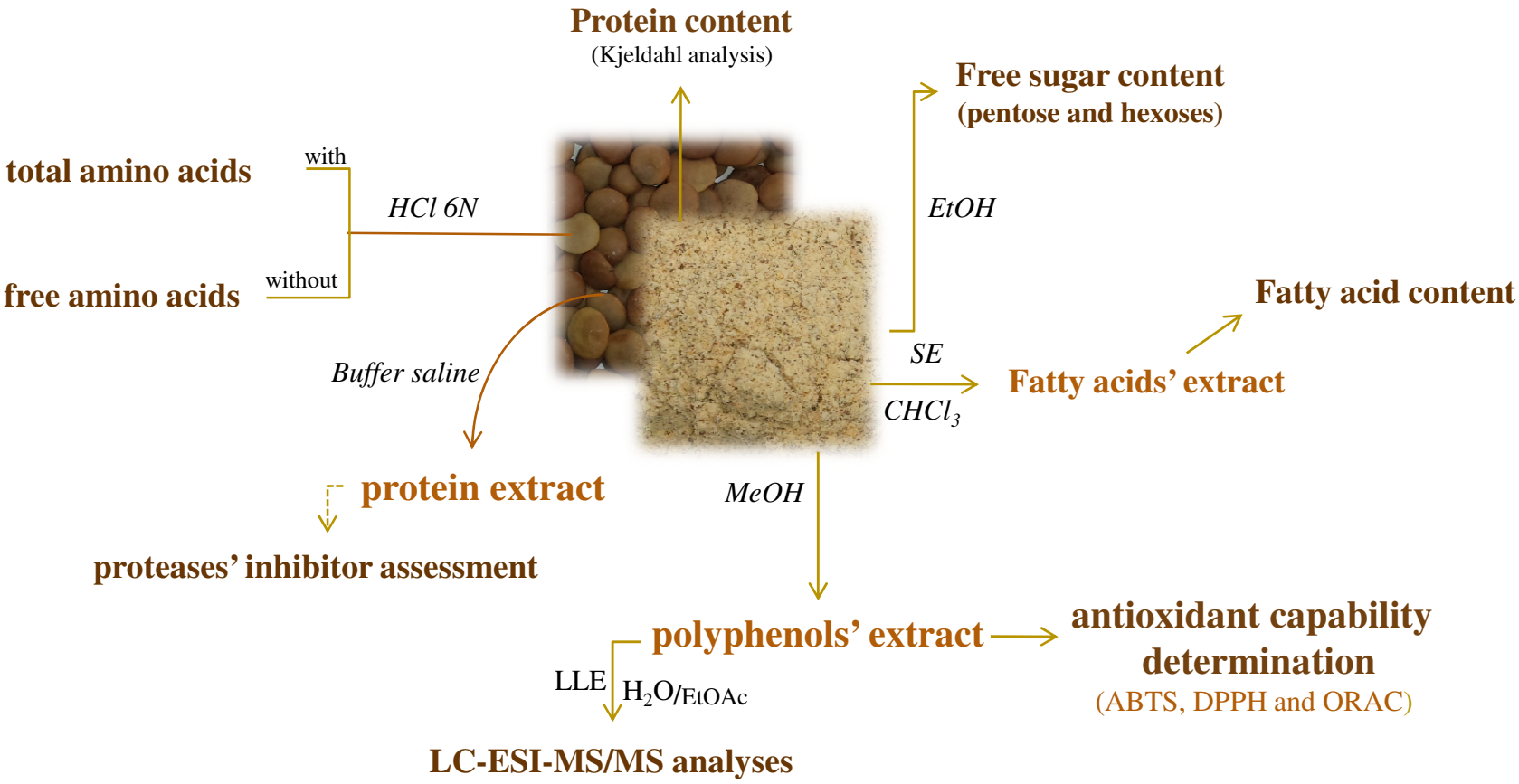
	ABTS	DPPH	ORAC	TPC
ABTS	1.000	0.977	0.997	0.907
DPPH		1.000	0.991	0.976
ORAC			1.000	0.938
TPC				1.000

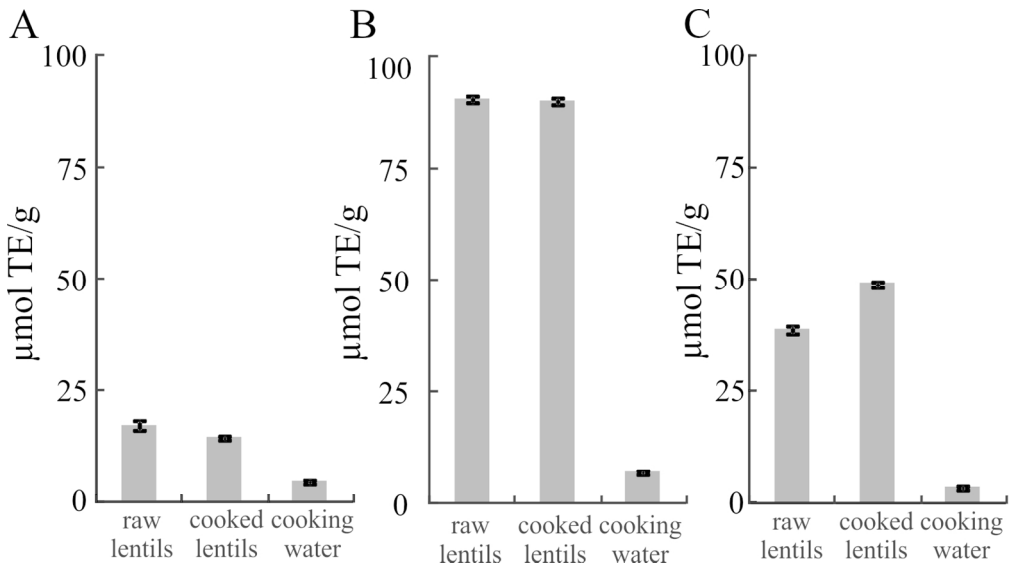
B

	ABTS	DPPH	ORAC	TPC	RACI
raw lentils	0.670	0.870	0.680	0.644	0.716
cooked lentils	0.186	0.862	0.358	1.061	0.617
cooking water	-1.465	-0.852	-1.486	-0.801	-1.151

Table 5. LC-MS/MS data of the main constituents of 'Valle Agricola' lentil seed extracts. LSE = raw Lentil Seed Extract; BLSE = Boiled Lentil Seed Extract; BH_2O = Boiling water

Peak	tR (min)	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)	Tentative assignment	LSE	BLSE	BH_2O
1	8.0	827	665, 503, 341, 179	pentahexoside	+	-	+
2	8.1	517	193, 179, 161	dihexosyl cyclitol (e.g. ciceritol)	+	-	+
3	8.2	665	503, 383, 341, 283, 231, 179	tetrasaccharide	+	-	+
4	8.5	503	341, 203, 163	trisaccharide (e.g. raffinose)	+	-	+
5	8.6	341	203, 163	disaccharide (e.g. galactinol)	+	-	+
6	22.6	901	755, 417, 285	kaempferol tetraglycoside	+	-	+
8	23.4	463	301	quercetin hexoside	+	+	-
7	25.9	477	301	quercetin hexuronide	+	+	+
9	26.0	755	593, 285, 284	kaempferol triglycoside	+	-	+
10	26.5	577	289, 225, 203, 163	procyanidin dimer	+	+	+
11	26.9	755	285, 284	kaempferol triglycoside	+	-	+
12	27.2	577	289, 225, 203, 163	procyanidin dimer	+	+	+
13	27.6	447	285	kaempferol hexoside	+	+	-
14	28.2	417	285, 241, 151	kaempferol pentoside	+	+	-
15	28.9	289	245, 137	catechin	+	-	-
16	29.5	289	245, 161, 137	epicatechin	+	-	-
17	30.2	301	273, 245, 229, 179, 173, 163, 151, 149, 121, 107	quercetin	+	-	-
18	30.8	341	179, 163	caffeoyl hexose	+	-	-
19	31.8	339	193, 163	feruloyl deoxyhexose	+	-	-
20	32.9	193	177,163	ferulic acid	+	-	-
21	36.8	305	289, 249, 219, 175, 147, 145, 131,119, 103	gallocatechin	+	-	-
22	37.7	305	289, 249, 219, 175, 147, 145, 131,119, 103	epigallocatechin	+	-	-





138x76mm (300 x 300 DPI)

Graphical abstract:

Valle Agricola lentil, an unknown lentil (*Lens culinaris* Medik.) seed from Southern Italy as a novel antioxidant and prebiotic source