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1 **Finding valuable bioactive components from Jerusalem artichoke (*Helianthus tuberosus* L.)**
2 **leaf protein concentrate in a green biorefinery concept**

3
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19 # Abstract

20 Jerusalem artichoke is widely known for its inulin-enriched tubers. Recently the opportunity has
21 been arisen to involve the whole plant in biorefinery concept due to its high lignocellulose
22 biomass and tuber production. This paper focuses on the repeatedly harvestable green biomass of
23 Jerusalem artichoke. Ultra-High Performance Liquid Chromatography-Electrospray
24 Ionization/Mass Spectrometry (UHPLC-ESI-MS) was applied to identify the phytochemicals in
25 Jerusalem artichoke leaf protein concentrate (JAPC) thermally extracted from green biomass of
26 three clones, i.e., Alba, Fuseau and Kalevala. Amino acid and fatty acid profiles as well as yield
27 of JAPC were also analyzed. The UHPLC-ESI-MS analyses showed that no toxic phytochemicals
28 were identified in JAPC. The results revealed, also, that JAPC is not only essential-amino acids-
29 rich but also contains substantial amounts of polyunsaturated fatty acids (66-68%) such as

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linolenic and linoleic acids. Linolenic acid represented 39-43% of total lipid content; moreover, the ratio between ω-6 and ω-3 essential fatty acids in JAPC was ~0.6: 1. Using UHPLC-ESI-MS, the following hydroxylated methoxyflavones were for the first time identified in JAPC, i.e., dimethoxy-tetrahydroxyflavone, dihydroxy-methoxyflavone, hymenoxin and nevadensin. These compounds are medically important since they are referred to as anti-cancer, anti-inflammatory and antioxidants. Also, liquiritigenin - estrogenic-like compound - was identified in JAPC alongside the following terpenes, i,e., loliolide and dihydroactinidiolide. However, no remarkable differences of phytochemicals, fatty acids and amino acids composition were seen among Jerusalem artichoke clones. The green biomass of tested clones ranged between 5 to 5.6 kg m^{-2} and JAPC yield varied from 28.6 to 31.2 g DM kg^{-1} green biomass with total protein content, on average, of 33.3%. According to our knowledge, this paper is the first scientific report highlighting bioactive substances in JAPC such as PUFA phytochemicals. These results clearly prove that JAPC is a valuable product which can direct towards human and animal nutrition as well as it can serve as basic material for different industrial purposes.

Keywords: Jerusalem artichoke; leaf protein concentrate; polyunsaturated fatty acids; phytochemicals; amino acids;; UHPLC-ESI-MS

Introduction

Ensuring an adequate supply of protein has become one of the challenges facing the world today, which is expected to worsen in the future as a result of the terrible increase in population and the erosion of agricultural land. The global protein supply relies on vegetal sources (57%), meat (18%), dairy products (10%), fish and shellfish (6%) and other animal products (9%), respectively [1]. Depending on species different organs of plants can serve as protein source such

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52 as seed (e.g. soy; pea; almond; pea; rice; wheat as seed-based protein), fruit (e.g. cranberry fruit-
53 based protein), leaf (e.g. moringa leaf-based protein) and root (e.g. maca root-based protein)
54 [2,3]. Recently, leaf-based protein has gained an intensive attention. Alfalfa and grasses are the
55 most perspective species in continental climate zone. Alfalfa protein-xanthophyll concentrate
56 (APC) is an extensively studied processed product of fractionated green biomass. It has already
57 manufactured in different countries such as France for feed and food purposes [4,5].

58 In context to leaf-based protein, the concept of green biorefineries is not be bypassed. Green
59 biorefineries are novel technology systems for production of materials and energy processing
60 using parts or total green plants [6]. Above all, green biorefinery technologies are based on
61 traditional technologies of green forage preservation, leaf-protein extraction, chlorophyll
62 production, and modern biotechnological and chemical conversion methods [6,7,8,9]. Sugar beet,
63 clover, alfalfa and grass are the most common and perspective species for green biorefinery
64 purposes in continental climate zone. However, several other crops may also be suitable.

65 Jerusalem artichoke can grow normally under harsh conditions [10,11]. It is tolerant to many
66 biotic and abiotic stresses such as pests and diseases [12], saline and alkaline soils, poor and
67 sandy soils with nearly zero fertilizer requirements [13], marginal lands [10], drought and high
68 temperature without interfering with other commercial crops as well [14]. Another important
69 advantage of Jerusalem artichoke, in comparison with the other fodder crops, is its ability to
70 produce huge green biomass under low input conditions (about 120 tons ha^{-1} fresh weight) [15].
71 This is an important aspect to avoid competition with the production of food in arable lands [16].
72 The nutritional value of Jerusalem artichoke is mainly due to the high inulin and fructose content
73 of sweetish tubers. Tuber, also, contains protein, nutrients and vitamins; therefore, it is valuable
74 for animal feeding and human consumption [16]. Although the aerial part of Jerusalem artichoke

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75 has widely gained the attraction of many researchers for bioethanol production due to its
76 lignocellulose content [12,17], it can be directed towards other significant uses such as animal
77 feeding as fresh forage, silage, or food pellets [16]. In addition, there are some information about
78 green leafy shoot which contain protein (stalk: 1.6%–4.5% DW; leaves 7.1%–24.5% DW),
79 volatile sesquiterpenes, some phenolic compounds, chlorophylls, carotenoids [11,17,18].
80 However, direct consumption of fresh or dried Jerusalem artichoke biomass is not preferable
81 because trichomes covered leaves and stem [11]. Alternatively, green biomass can be fractionated
82 mechanically to green juice and fiber fractions. The cell wall-deprived green juice can be
83 thermally treated in order to extract proteins by coagulation. From the aspect of green biorefinery
84 the separate leaf protein concentrate as main product has special importance hence to become
85 competitive process it should produce at least one product of high value (such as a high value
86 chemical or material).

87 In accordance with the above, the present study aimed to provide a detailed insight into the
88 extraction efficiency and biochemical composition of green biomass originated Jerusalem
89 artichoke leaf protein concentrate (JAPC). Three clones of Jerusalem artichoke representing
90 different climatic zones were grown under low input conditions in Hungary. Biochemical
91 composition and qualitative determination of phytochemicals in JAPC were conducted via Ultra-
92 High Performance Liquid Chromatography-Electrospray Ionization/Mass Spectrometry
93 (*UHPLC-ESI-MS*).

94 Materials and Methods

95 Experimental installation

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96 A field experiment was conducted in 2016 at the Horticultural Demonstration garden of the
97 University of Debrecen, Hungary (47°33'N; 21°36'E). Three different clones of Jerusalem
98 artichoke (i.e., Alba, Fuseau and Kalevala) were compared for their fresh aerial biomass yield,
99 JAPC and phytochemical composition under low input conditions. Tubers of Jerusalem artichoke
100 clones representing three climatic zones were obtained from different sources as follow: Alba
101 was brought from Hungarian market; Fuseau was brought from Ismailia, Egypt; and Kalevala
102 was obtained from Helsinki, Finland. The experiment was set up in a randomized complete block
103 design with six replicates. The area of the experimental plot was 0.8×0.6 m^2; the row was 3.5 m
104 in length and 0.8 m in width within-row spacing 0.6 m. The cultivation of the Jerusalem artichoke
105 clones started on 5th April 2016 using size-identical tubers (60 – 80 g/tuber). No irrigation and
106 fertilization were applied in the plantation during the growing season. Chemical characteristics of
107 the experimental soil were: total N (555±2 mg kg^{-1}); total P (6793±17 mg kg^{-1}); total K (1298±7
108 mg kg^{-1}) and humus (1.9±0.02%).

109 Harvest of aboveground fresh biomass

110 Considering the re-growing ability of Jerusalem artichoke plants, the green biomass of the three
111 clones was harvested two times during the growing season when young shoot reached 1.3 - 1.5 m
112 height from soil surface. The first harvest was carried out on 27th June 2016, while the second
113 harvest was on 8th August 2016. Fresh yield of aerial part was measured.

114 Fractionation of harvested green biomass

115 The harvest of Jerusalem artichoke plants was carried out in the early morning and immediately
116 transferred to the laboratory in an ice box to inhibit the degradation chemical compounds. Plants
117 were harvested 20 cm above soil surface. One-kilogram green biomass was mechanically

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118 pressed and pulped by a twin-screw juicer (Green Star GS 3000, Anaheim, Canada) to green juice
119 and fiber fractions in three replicates. Thereafter, the green juice was thermally coagulated at 80
120 °C to obtain JAPC. The JAPC was separated from brown-colored liquid fractions using cloth
121 filtration. Both fresh and dry masses of JAPC were weighted. The JAPC was lyophilized using
122 the Alpha 1-4 LSC Christ lyophilizer.

123 Biochemical composition of JAPC

124 Crude protein content

125 Total protein content of JAPC was measured as total N content by Kjeldahl method [19] as
126 follows: one gram lyophilized sample was weighted in a 250 mL Kjeldahl digestion tube and then
127 15 mL concentrated sulfuric acid (99%, VWT, Ltd) and 2 catalyst tablets were added. The
128 Kjeldahl digestion tubes were transferred to Tecator Digestor (VELT, VWR Ltd.) at 420°C for
129 1.5 hour. The total N content in the digested samples was later measured by titration method; and
130 total N content of the sample was calculated based on the weight of the titration solution and the
131 sample weight. The total protein content of the sample was calculated as follow: Total protein, %
132 = total N content × 6.25.

133 Quantification of amino acid composition in JAPC by Amino acid analyzer

134 For the sample preparation lyophilized and grinded sample of JAPC was digested with 6M HCl
135 in at 110°C for 23 hours. Since the digested sample should contain at least 25 mg N, therefore the
136 measured weight of the samples was variable. For removing the air from the samples inert gas
137 and vacuum alternating with applying a three-way valve were used. After hydrolyzing, the
138 sample was filtered into evaporator flask. The filtrate was evaporated under 60°C to achieve
139 syrup-like consistency. Thereafter, distilled water was added to the sample and it was evaporated

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140 again using the same circumstances. This procedure was repeated one more time. The evaporated
141 sample was washed and completed with citrate buffer pH 2.2. For the analysis of amino acid
142 composition INGOS AAA500 (Ingos Ltd., Czech Republic) Amino Acid Analyzer was used. The
143 method of separation based on ionic exchange chromatography with postcolumn derivatization of
144 ninhydrine. UV/VIS detector was used on 440/570 nm.

145 Determination of fatty acid composition in JAPC by Gas Chromatography

146 Esterification of fatty acids in JAPC fraction into methyl esters was conducted using sodium
147 methylate catalyst. Seventy milligram of lyophilized homogeneous sample was weighed into a 20
148 mL tube; 3 mL of n-hexane, 2 mL of dimethyl carbonate and 1 mL of sodium methylate in
149 methanol were added. The contents of the test tube were shaken for 5 minutes (Janke and Kunkel
150 WX2) and then 2 mL of distilled water was added and shaken again. The samples were then
151 centrifuged at 3000 rpm for 2 minutes (Heraeus Sepatech, UK). The 2.0 mL of supernatant
152 (hexane phase) was transferred into container through a filter paper, which contained anhydrous
153 sodium sulfate. The prepared solution contained approximately 50-70 mg cm^{-3} fatty acid methyl
154 ester (FAME) was directly suitable for gas chromatography measurement. Gas Chromatography
155 analysis was performed by Agilent 6890 N coupled with an Agilent flame ionization detector. A
156 Supelco Omegawax capillary column (30 m, 0.32 mm i.d., 0.25 µm film thickness) was used to
157 separate FAMEs. The oven temperature was 180 °C and total analysis time was 36 min. An
158 Agilent 7683 automatic split/splitless injector was used with 280 °C injector temperature and
159 100:1 split ratio. Injection volume was 1µL. The carrier gas was hydrogen with a flow rate of 0.6
160 ml $min^{-1}$and the makeup gas was nitrogen with a flow rate of 25.0 ml min^{-1}. The components
161 were identified by retention data and standard addition.

162 Screening of phytochemicals in JAPC by UHPLC-ESI-MS

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163 **Sample preparation**

164 For the hydro-alcoholic extracts 0.5 g grinded JAPC powder was used and extraction was done
165 by 25 mL methanol:water solution at ratio of 7:3. The mixture was stirred on 150 rpm for 2h at
166 room temperature. The hydro-alcoholic extracts were filtered using a 0.22 μm PTFE syringe
167 filter.

168 **UHPLC-ESI-MS analysis**

169 The phytochemical analyses were performed by UHPLC-ESI-MS (Ultra-High Performance
170 Liquid Chromatography-electrospray ionization/mass spectrometry) technique using a Dionex
171 Ultimate 3000RS UHPLC system (Thermo Fisher, USA) coupled to a Thermo Q Exactive
172 Orbitrap hybrid mass spectrometer equipped with a Thermo Accucore C18 analytical column (2.1
173 mm × 100 mm, 2.6 μm particle size). The flow rate was maintained at 0.2 mL/min. The column
174 oven temperature was set to 25°C ±1 °C. The mobile phase consisted of methanol (A) and water
175 (B) (both were acidified with 0.1% formic acid). The gradient program was as follows: 0 - 3 min,
176 95 % B; 3 - 43 min, 0 % B; 43 - 61 min, 0% B; 61 - 62 min, 95% B; 62 - 70 min, 95% B. The
177 injection volume was 2 μL.

178 **Mass spectrometry (MS) conditions**

179 Thermo Q Exactive Orbitrap hybrid mass spectrometer (Thermo Fisher, USA) was equipped with
180 an electrospray ionization (ESI) source. The samples were measured in positive and negative
181 ionization mode separately. Capillary temperature: 320 °C. Spray voltage: 4.0 kV in
182 positive ionization mode and 3.8 kV in negative ionization mode, respectively. Resolution:
183 35,000 in the case of MS1 scans and 17,500 in the case of MS2 scans. 100-1500 m/z
184 was the scanned mass interval. For MS/MS scans the collision energy was

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185 set to 30 NCE. The difference between measured and calculated molecular ion
186 masses was less than 5 ppm in every case. The data were acquired and processed using Thermo
187 Trace Finder 2.1 software based on own and internet databases (Metlin, Mass Bank of North
188 America, m/z Cloud). After processing the results were manually checked using Thermo
189 Xcalibur 4.0 software. The compounds found in the extracts were identified on the basis our
190 previous published works or data found in literature using exact molecular mass, isotopic pattern,
191 characteristic fragment ions and retention time.

192 Quality assurance of results

193 Glassware and plastic ware for analyses were usually new and were cleaned by soaking in 10%
194 (v/v) HNO_3 for a minimum of 24 h, followed by thorough rinsing with distilled water. All
195 chemicals were analytical reagent grade or equivalent analytical purity. All the used equipment
196 was calibrated and uncertainties were calculated. Internal and external quality assurance systems
197 were applied in the Central Laboratory of the University of Debrecen according to MSZ EN ISO
198 5983-1: 2005 (for Total N) and Bunge Private Limited Company Martfű Laboratory MSZ 190
199 5508: 1992 (for fatty acid composition).

200 Statistical analysis

201 The experimental design was established as a randomized complete block design with six.
202 Results of the experiments were subjected to one-way ANOVA by 'R-Studio' software and the
203 means were compared by Duncan's Multiple Range Test at $p < 0.05$ [20].

204 Results

205 Green biomass of Jerusalem artichoke clones

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206 Results of aboveground fresh biomass yield presented in Table 1. It is showed that the yield of
207 Jerusalem artichoke clones were similar. No significant differences among tested clones (i.e.,
208 Alba, Fuseau and Kalevala) were noticed especially during the 1st harvest. By contrast, the
209 harvesting time influenced the yield. Significant lower yield could be harvested at the second
210 harvest time. The average fresh biomass yield was approximately 5.3 kg m^{-2} in the 1st harvest,
211 while in the 2nd harvest the yield significantly reduced as the average biomass was 2.4 kg m^{-2}
212 (Table 1). The calculated total aboveground fresh biomass yield – as an average – was estimated
213 to be 7.7 kg m^{-2}.

214 **Table 1.** Aboveground fresh biomass, dry mass and total protein content of Jerusalem artichoke
215 leaf protein concentrate (JAPC) isolated from green biomass of different clones

216

217 JAPC yield

218 Results of JAPC isolated from 1 kg fresh green biomass of Jerusalem artichoke clones by thermal
219 coagulation are displayed in Table 1. Statistical analysis showed insignificant differences among
220 Jerusalem artichoke clones whether in 1st or 2nd harvests. The JAPC yield ranged from 28.3
221 (Fuseau) to 32.3 (Kalevala) g kg^{-1} fresh biomass in the 1st harvest, while in the 2nd harvest JAPC
222 yield varied from 28 (Kalevala) to 30.4 (Alba) g kg^{-1} fresh biomass (Table 1). However, results
223 showed that the average JAPC dry yield of the 1st and 2nd harvests were 30.8 and 29.1 g kg^{-1} fresh
224 biomass, respectively. Therefore, it could be calculated that 1 kg of green biomass of Jerusalem
225 artichoke gives approximately 30 g JAPC dry mass as an annual average.

226 Total protein content of JAPC

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227 The total protein content (m/m%) of JAPC generated from fresh green biomass of Jerusalem
228 artichoke clones ranged between 33.3 m/m% (Fuseau) and 35.3 m/m% (Alba) in the 1st harvest,
229 while in the 2nd harvest it varied from 31.6 m/m% (Alba) to 35.2 m/m% (Fuseau). Statistically,
230 no significant differences were calculated either between clones or between harvests (Table 1).
231 The average total protein content in the 1st harvest was 34.1 m/m% and in the 2nd harvest was
232 33.4 m/m%. The annual average of total protein content of JAPC extracted from Jerusalem
233 artichoke fresh biomass is estimated to be 33.8 m/m% (Table 1).

234 Quantitative analysis of amino acid composition of JAPC

235 The results of amino acid composition of JAPC obtained from green biomass of Jerusalem
236 artichoke clones are presented in (Table 2). The essential amino acids (i.e., lysine, histidine,
237 isoleucine, leucine, phenylalanine, methionine, threonine and valine) play a major role in feeding;
238 therefore, they receive a special interest. Among the examined Jerusalem artichoke clones,
239 Kalevala possessed the highest values of 5 out of the 8 essential amino acids (i.e., phenylalanine,
240 histidine, isoleucine, threonine and valine). Also, aspartic acid, glycine, glutamic acid, proline
241 and serine had the highest values 4.23, 2.13, 4.82, 2.20 and 1.90 m/m%, respectively, in JAPC of
242 Kalevala (Table 2). The concentration of lysine as essential amino acid has special importance in
243 animal feeding. We found that the lysine content of Alba, Fuseau and Kalevala (ranged between
244 2.19 to 2.32 m/m%, in the first harvest. The tendency of change in lysine content was similar but
245 with higher values in the second harvest considering the Alba, Fuseau, Kalevala as their content
246 varied from 2.35 to 2.54 m/m%. The methionine is another limiting amino acid. The methionine
247 content of Alba and Fuseau clones changed between 0.82 to 0.95 m/m% in both harvests (Table
248 2). Results, also, showed that lysine content increased in the 2nd harvest compared to 1st harvest

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249 for all clones; while, a reduction in methionine content was found in the 2nd harvest compared to
250 1st harvest for all clones except Fuseau.

251 **Table 2.** Amino acid profile (%) of Jerusalem artichoke leaf protein concentrate (JAPC)
252 extracted from green biomass of different clones

253

254 Quantitative analysis of fatty acid composition of JAPC

255 Both saturated (SFA) and unsaturated fatty acids (UFA) could be detected from originated JAPC.
256 Polyunsaturated fatty acids (PUFA) including linoleic acid (C18:2ω –6), linolenic acid (C18: 3ω
257 –3) and arachidonic acid (C20:4ω –6) were the predominant fatty acids (66 - 68%) in all
258 Jerusalem artichoke clones (Figs 1 and 2). Among them linolenic acid (38.6 – 42.7%) exhibits a
259 narrow range of distribution with highest values regardless of clones or harvesting time. The
260 Linoleic acid (C18:2ω –6) had the second highest concentration with min. 23.4 % in 1st harvest
261 JAPC of Kalevala and with max. 26.9 % in 2nd harvest JAPC of Alba. All analyzed JAPC had the
262 lowest proportion of arachidonic acid (C20:4ω –6) at 0.3 – 0.6% of total fatty acids (Fig1).
263 Among monosaturated fatty acid (MUFA) the oleic acid (C18:1ω–9) could be detected with
264 higher values (6.6 – 11.6%). The proportion of palmioletic acid (C16:1ω–7) was significant
265 lower, changed in the range of 0.7 – 1.1% (Fig 3). From saturated fatty acids (SFA) myristic acid
266 (C14:0), palmitic acid (C16:0) and stearic acid (C18:0) could be separated. Palmitic acid (C16:0)
267 was is the most abundant saturated component with no significant differences (16.4 – 17.9%)
268 considering the clones and harvesting time. The percentage composition of myristic acid (2.5 –
269 6.9%) and stearic acid (1.5 – 1.8%) in JAPC fractions were markedly lower compare to palmitic
270 acid (Fig 1).

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271 **Fig 1.** Fatty acid composition (%) of Jerusalem artichoke leaf protein concentrate (JAPC)
272 extracted from green biomass of different clones (Alba, Fuseau and Kalevala).

273 **Fig 2.** Distribution of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and
274 polyunsaturated fatty acids (PUFA) in Jerusalem artichoke leaf protein concentrate
275 (JAPC) extracted from green biomass of different clones (Alba, Fuseau and Kalevala).

276

277 Reverse tendency of oleic acid and myristic acid content could be found considering the first and
278 second harvest. Myristic acid content was higher in the 1st harvest of JAPC of all three Jerusalem
279 artichoke clones. While the oleic acid content was higher in the 2nd harvest of JAPC of Alba and
280 Kalevala clones (Fig1).

281 Screening of phytochemicals of JAPC by UHPLC-ESI-MS

282 The identification of compounds was primarily based on the correspondence of the ion from the
283 deprotonated or protonated molecule using scientific literature results and fragmentation patterns
284 of similar compounds. Profiles of phytochemicals of JAPCs isolated from different Jerusalem
285 artichoke clones (i.e., Alba, Fuseau and Kalevala) showed negligible differences. Up to 61
286 phytochemicals were defined based on specific retention time, accurate mass, isotopic
287 distribution and fragmentation pattern, and by screening MS databases like Metlin, mzCloud and
288 Massbank (Table 3). Table 3 showed that the phenolic compounds were significant part of the
289 identified compounds. Regardless of Jerusalem artichoke clones, all three caffeoyl quinic acid
290 isomers could be identified in the JAPCs with a characteristic $[M-H]^-$ ion at m/z 353.0873
291 measured by LC-ESI-MS technique in present experiment. In accordance with Yuan et al. [21] 3-
292 O-Caffeoylquinic acid was in highest ratio, while neochlorogenic and chryptochlorogenic acids
293 were in fewer amounts (Fig 3). Along with it, we could identified three di-O-caffeoylquinic acid
294 isomers exhibited $[M-H]^-$ ion at m/z 515.1190, four Coumaroylquinic acid isomers $[M-H]^-$ ion

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295 at m/z 337.0924 and a 5-O-Feruloylquinic acid $[M-H]^-$ ion at m/z 367.1029 from hydro-
296 alcoholic JAPC extracts. The measurement also revealed the existence of a compound with a
297 $[M-H]^-$ ion at m/z 299.0767 in all JAPC extracts. The ion scan experiment of this ion showed the
298 corresponding fragment ions at m/z 137.0233; 113.0229; 93.0331; 85.0281 and 71.0122. After
299 comparison with database this compound was identified as salicylic acid-O-glucoside.

300 **Table 3.** Chemical composition of Jerusalem artichoke leaf protein concentrate (JAPC) extracted
301 from green biomass

302 **Fig 3.** Extracted Ion Chromatograms (XIC) and MS spectra of selected phytoconstituents from
303 Jerusalem artichoke leaf protein concentrate: A: quercetin- 3-O-glucuronide; B:
304 Scopoletin; C: Loliolide; D: Liquiritigenin

305

306 Among flavonoids quercetin-O-glucoside with m/z 463.0877, isorhamnetin-3-O-glucoside with
307 m/z 477.1033, kaempferol glucuronide with m/z 461.0720 and kaempferol-3-O-glucoside with
308 m/z 447.0927 were found in all studied JAPC in agreement with Jerusalem artichoke related
309 literature [18,22]. However, according to our knowledge this is the first time to identify
310 glucuronide derivatives of isorhamnetin and quercetin (Table 3 and Figs 3 and 4). In addition to
311 flavonols, most of the identified flavonoids are belonged to flavones. None of them have been
312 described yet from *Helianthus tuberosus* according to our knowledge. All of identified flavones
313 contained one or more methoxy groups besides hydroxyl groups. For instance we could identified
314 two dimethoxy-trihydroxyflavone isomers exhibited $[M-H]^-$ ion at m/z 329.0661; dimethoxy-
315 tetrahydroxyflavone $[M-H]^-$ ion at m/z 345.0611; dihydroxy-methoxyflavone $[M-H]^-$ ion at m/z
316 283.0607, trihydroxy-trimethoxyflavone $[M-H]^-$ at m/z 359.0767. Hymenoxin (5,7,Dihydroxy-
317 3',4',6,8-tetramethoxyflavone) at m/z 375.1080 and Nevadensin (5,7-Dihydroxy-4',6,8-
318 trimethoxyflavone) at m/z 317.1389; however, could be identified in positive ESI mode (Table 2

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319 and Fig 2). Liquiritigenin (4',7-Dihydroxyflavanone) $[M-H]^-$ at m/z 255.0657 was the only
320 flavanone found in all studied JAPC of Jerusalem artichoke clones.
321 Besides polyphenols three different terpenes were consistently appeared in all studied JAPC of
322 Jerusalem artichoke clones. Loliolide as C_{11} monoterpenoid lactone was one of them exhibited
323 $[M+H]^+$ ion at m/z 197.1178. As well as dihydroactinidiolide as a volatile monoterpene $[M+H]^+$
324 ion at m/z 181.1229 and 7-Deoxyloganic acid isomer as iridoid monoterpene $[M-H]^-$ ion at m/z
325 359.1342 were defined. Several proteinogen aminoacids (Table 3) could be identified. Among
326 vitamins we were able to identify some vitamin B such as nicotinic acid (niacin) $[M+H]^+$ ion at
327 m/z 124.0399 and riboflavin $[M+H]^+$ ion at m/z 377.1461. In addition, organic acids (i.e., malic
328 acid and citric acid) and plant hormones (i.e., indole acetic acid) could also be identified in
329 Jerusalem artichoke JAPC.
330 **Fig 4.** Extracted Ion Chromatoram of chlorogenic acid isomers
331

332 Discussion

333 Increasing the global demand for protein and with limited soy cultivation along with reliance on
334 many countries in imports; all make the search for alternatives a necessity. Hence, recently the
335 interest in green biomass has been gained a wide attraction, as it may represent a relevant
336 substitute of seed-based proteins. Jerusalem artichoke proved its ability to grow on a wide range
337 of soils with low inputs requirement. Moreover, it is tolerant to many biotic and abiotic stresses
338 such as insects, drought, heat and salinity [17]. For instance, in the harsh environments, it yields
339 around 30 Mg ha^{-1} DM forage (aerial part) with 15.3% of crude protein [17]. These qualities
340 make Jerusalem artichoke a suitable candidate for a sustainable JAPC production with no
341 competition with the recognized fodder crops. Therefore, three Jerusalem artichoke clones

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342 representing three different climatic zones, such as humid continental (Hungary), arctic (Finland)
343 and hot desert climate (Egypt), were assessed for their JAPC production from a rain-fed
344 plantation with zero fertilizers.

345 Chloroplastic and cytoplasmic proteins of plant cells are the main source for JAPC production,
346 and they found at higher concentration in leaf tissues than in stem [23]. Young stems and leaves
347 of Jerusalem artichoke are succulent and turn to be woody with the time [15] therefore, in the
348 present study we harvested the aerial part two times during the growing season when reached 1.3
349 - 1.5 m height, respectively. The fresh biomass aerial part of Jerusalem artichoke clones in
350 average was 5.3 kg m^{-2} in the first harvest and reduced to 2.4 kg m^{-2} in the 2nd harvest. However,
351 the total measured fresh biomass of the aboveground part was 7.7 kg m^{-2}; no significant
352 differences among studied clones were reported. Non-native varieties (Fuseau and Kalevala)
353 displayed high green biomass productivity under Hungarian climate. With average moisture
354 content of 47.8%, it was calculated that total dry shoot biomass was 3.7 kg m^{-2} which is
355 equivalent to 36.8 Mg ha^{-1}. This total dry biomass of aerial part was higher than this cited by
356 [23], who reported 25 Mg ha^{-1}. It is important to be cognizant of the fact that this green biomass
357 was yielded from rain-fed plantation of Jerusalem artichoke; therefore, Jerusalem artichoke is
358 expected to produce higher aerial biomass yield since its shoot part is more sensitive to water
359 stress than tuber. According to [24] the irrigated plots of Jerusalem artichoke had higher
360 aboveground biomass by 98% than unirrigated plots.

361 Using aerial part of Jerusalem artichoke for animal feeding as fresh fodder or processed either in
362 the form of silage or feed formulations has long been known. However, this could be restricted
363 by trichomes which cover the surface of leaves and stem, as well as decreased protein and
364 increased lignin contents when plants become older [15,25]. Hence, processing extracts the

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365 protein from shoot part in the form of JAPC is vital for increasing the economic value of
366 Jerusalem artichoke. Another reason for the importance of JAPC production is that fresh stem is
367 lower quality for feeding plants since it contains higher carbohydrate content and lower protein
368 content than leaves [26]. Pressing and pulping of collected fresh green biomass resulted in, as an
369 average, 10.2% fresh mass of JAPC. These results emphasize that Jerusalem artichoke has the
370 qualities of what makes it suitable candidate for JAPC production.

371 Average total protein content of JAPC generated from Alba, Fuseau and Kalevala clones was
372 33.4 m/m%. However, most of isolated protein is referred to leaves as Jerusalem artichoke leaves
373 contain three times higher total protein than stem [27]; our results were in agreement with these
374 findings. Total protein content of JAPC extracted mainly from leaves most of which is made up
375 of assimilation parenchyma tissue (80- 87%) with easily released cytoplasmic and chloroplast
376 proteins. Rubisco has the greatest significance among leaf soluble protein with its high nutritional
377 value [28]. Harvesting time of aerial part is critical from the aspects of quantity and quality of
378 JAPC production. Rashchenko [29] reported that nitrogen content in older leaves decreased by
379 approximately 50% compared to young leaves. Seiler [30], also, reported that total protein
380 reduced by 32.6% from vegetative to flowering stage of Jerusalem artichoke plants. Knowing
381 this, the shoots were harvested in the maximum green leaf state (1.3 – 1.5 m) ahead of senescence
382 and avoiding the dryness of bottom leaves. Following this thread, the results showed no big
383 difference in protein content between the two harvests.

384 When it comes to the ideal protein source, the amino acid profile cannot be ignored because
385 among the 20 proteinogenic amino acids nine cannot be synthetized by most animal species [31].
386 The ratio of these essential amino acids has special interest. Among green biomass originated
387 fractions, JAPC as dedicated protein enriched product for feed was examined more thoroughly.

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388 Several indispensable amino acids i.e., lysine, isoleucine, leucine, methionine and threonine
389 showed high content in JAPC. However, higher contents of amino acids in JAPC were reported
390 by [23]; this could be attributed to the different extraction method and different varieties. The
391 results of amino acids exhibited minor measured differences between the two harvests; this could
392 be due to the different weather and plant age as has been previously documented [15,29,30].

393 Several literatures discussed the fatty acid and lipid content in tubers of Jerusalem artichoke
394 [15,32]; however, meager information about fatty acid composition in leaves is available in
395 scientific studies. Nowadays, there is a growing attention in polyunsaturated fatty acids (PUFA)
396 because humans and other mammals are incapable to synthesize omega-6 and omega-3 PUFA in
397 the lack of delta (Δ) 12 and Δ15 desaturase enzymes. These enzymes are responsible to insert cis
398 double bond at the n-6 or n-3 positions [5]. Hence, linolenic acid (C18: 3ω –3) and linoleic acid
399 (C18:2ω –6) are essential nutrients convert from oleic acid in the endoplasmic reticulum (ER) of
400 plant cells. Linolenic acid is the precursor of longer-chain PUFA such as eicosapentaenoic acid
401 (EPA: C20:5ω –3) and docosahexaenoic acid (DHA: C22:6ω –3) which can be synthesized in
402 human. Similarly linoleic acid (C18:2ω –6) is also essential precursor to synthesize dihomo- γ-
403 linoleic acid (DGLA: C20:3ω –6) and arachidonic acid (AA: C20:4ω –6). Because of their
404 essentiality, linolenic acid and linoleic acid need to be supplied with a diet of animals or humans.
405 Regardless of observed clones the highest contribution to the fatty acid profile were noted for
406 linolenic acid (C18: 3ω –3) with 38.6 – 42.7% values and linoleic acid (C18:2ω –6) with 23.4 –
407 26.9 % in JAPCs (Fig 1). Along with anthropological and epidemiological studies right
408 proportion of linoleic acid and linolenic acid should be emphasized. The ratio of omega-6 to
409 omega-3 essential fatty acids evolved ~1: 1 in the evolutionary history of human diet In contrast,
410 following the current Western diet the ratio has shifted to 10-20:1 which is not desirable from

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411 health aspect and promotes the pathogenesis of many diseases [33]. We found ~0.6: 1 ratio of
412 omega-6 to omega-3 essential fatty acids in JAPC which is very favorable, close to the
413 Paleolithic nutrition.

414 Arachidonic acid as a PUFA could also be measured from JAPC even at low proportion (0.3 –
415 0.6%) in agreement with Shanab et al. [34] who found little amounts of AA are in terrestrial
416 plants. Arachidonic acid mainly could be identified in many algal groups, which grow
417 photoautotrophically or heterotrophically. Among marine macroalgae AA can reach 60% of total
418 FAs content in case of *Gracilaria* sp. red alga.

419 The saturated palmitic acid (C16:0), stearic acid (C18:0) and the monosaturated form of stearic
420 acid the oleic acid (C18:1ω–9) are often referred to as common fatty acids. They biosynthesis
421 occurs in plastids and partially incorporated into cell and subcellular membranes [35]. The JAPC
422 originated mainly from crushed cells of vegetative tissues containing membrane debris. This
423 explains the relative higher proportion of palmitic acid (16.4 – 17.9%) and oleic acid (6.6 –
424 11.6%). Considering the harvesting time, oleic acid content of Alba and Kalevala JAPC showed
425 higher value in the 2nd harvest (when the nights were colder). According to Barrero-Sicilia et al.
426 [36] plants often respond to low temperature by increasing unsaturated fatty acids in membrane
427 along with increased membrane fluidity and stabilization. We found reverse tendency in saturated
428 myristic acid (C14:0) content of JAPC. It showed higher values in the 1st harvest (when the nights
429 were warmer) in case of all three Jerusalem artichoke clones.

430 The phytochemical screening by UHPLC ESI MS was performed in both negative and positive
431 ESI modes owing to the varying ionization requirements of compounds. Negative mode was used
432 for identification of flavonoid and phenolic acid (hydroxycinnamic acid and benzoic acid)
433 derivatives which provided better sensitivity. The easy protonation of nitrogen in positive mode

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434 made it suitable for identification of terpenes, amino acids and coumarins, coumarylquinic acids.
435 Within non-flavonoid phenolic constituents, the caffeoyl quinic acid as also called chlorogenic
436 acid isomers are important subgroup and their presence is characteristic of Asteraceae family.
437 According to some literature the chlorogenic acid (3-O-Caffeoylquinic acid) is the most abundant
438 isomer in plant sources, while the cryptochlorogenic acid (4-O-Caffeoylquinic acid) and
439 neochlorogenic acid (5-O-Caffeoylquinic acid) are in low concentrations [37]. Yuan et al. [21]
440 found 3-O-Caffeoylquinic acid and 1,5-dicaffeoyl quinic acid in the highest concentration in
441 Jerusalem artichoke extracted leaves. However, Liang and Kitts [38] reported that generally the
442 5-O-Caffeoylquinic acid is the predominant isomer in fruits and vegetables. The presence of
443 these phenolic acids is interesting from both human and animal aspects because of several
444 biological roles are attributed to caffeoyl quinic acid isomers in such as antioxidant activity,
445 antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, antipyretic, neuroprotective,
446 anti-obesity, antiviral, anti-hypertension, and a central nervous system stimulator. In addition,
447 they can be confirmed to modulate lipid metabolism and glucose in both genetically and healthy
448 metabolic related disorders [21,39]. Based on the health promoting effects, caffeoyl quinic acid
449 isomers are increasingly recommended as natural and safe food additive supplement instead of
450 synthetic antibiotics and immune boosters.

451 The presence of coumarins as non-flavonoid polyphenols has also been revealed from JAPC
452 fractions of Jerusalem artichoke. Scopoletin, isoscopoletin, were identified with a characteristic
453 $[M+H]^+$ ion at m/z 193,05, in addition 6-methylcoumarin and fraxidin were also found in
454 assessable quantities.

455 Flavonoids are widespread secondary metabolites within phenolic constituents in plant kingdom.
456 However, few of them were described in Jerusalem artichoke such as isorhamnetin glucoside,

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457 kaempferol glucuronide and kaempferol-3-o-glucoside from leaves [18]. In present study, 18
458 flavonoids were recognized in JAPC (Table 3). Generally, cell vacuoles are the main storage of
459 soluble flavonoids. Primarily, the solubility of flavonoids is due to the different sugar
460 substitution. Because of JAPC is coagulated pressed green juice - which mainly contain the
461 cytoplasm included vacuoles - this could be one of the reasons of the relatively more flavonoids
462 found in the present study. Among sugars glucose and glucuronic acid at a single position is
463 probably the most common substituents [40]. In screened JAPC, glycosylated flavonoids were
464 occurred. The importance of flavonoid glucuronides is related to health-promoting activities such
465 as anti-inflammatory and neuroprotective activities of quercetin-3-*O*-glucuronide [41].
466 Most of identified flavonoid compounds by UHPLC-ESI-MS technique belonged to the flavones.
467 All of identified flavones were hydroxylated methoxyflavone which mean one or more methoxy
468 groups on flavone basic framework besides/instead of hydroxyl groups. Substitution of a
469 methoxy group for the hydroxyl group in flavones has significant importance. One side the
470 hydroxyl groups flavones have free radical scavenging activity, but extensive conjugation of free
471 hydroxyl groups to flavones results in low oral bioavailability hence they undergo rapid sulfation
472 and glucuronidation in the small intestine and liver by phase II enzymes; consequently,
473 conjugated metabolites can be found in plasma not the original compounds [42]. However, if one
474 or more hydroxyl groups are capped by methylation, the substitution of a methoxy group by the
475 hydroxyl group induces an increase in metabolic stability, improves transport and absorption.
476 Considering the biological properties and chemical characteristics of hydroxyl and methoxy
477 groups together, the hyroxylated methoxyflavones combine many advantages of both functional
478 groups, improving their potential for application in human health [42]. Therefore, the several
479 hyroxylated methoxyflavones such as dimethoxy-trihydroxyflavone isomers; dimethoxy-
480 tetrahydroxyflavone; dihydroxy-methoxyflavone, trihydroxy-trimethoxyflavone, hymenoxin and

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481 nevadensin increase the value of JAPC. liquiritigenin (4',7-Dihydroxyflavanone) which has been
482 previously identified by Johansson et al. [17] in flower of *Helianthus tuberosus* was found in our
483 JAPC. The liquiritigenin is known as promising active estrogenic compound, it is highly selective
484 estrogen receptor β agonist. It might be helpful to women who suffer from the menopause
485 symptoms [43].

486 Three different terpenes were consistently appeared in all tested JAPCs of Jerusalem artichoke
487 clones. Loliolide as C_{11} monoterpenoid lactone is considered as a photo-oxidative or thermal
488 degraded product of carotenoids [44]. Similarly, we could identify dihydroactinidiolide as a
489 volatile monoterpenoid. It is a flavor component in several plants, such as tobacco and tea.
490 According to Yun et al. [45] thermal treatment induce the formation of dihydroactinidiolide from
491 β-carotene. Kaszás et al. [11] confirmed that the green juice contains markedly amount of
492 carotenoids which probably can partially convert to loliolide or dihydroactinidiolide as reason of
493 detected terpenes. Role of loliolide is confirmed by some scientific studies. According to these
494 studies, growth and germination inhibition, as well as phytotoxic activities were demonstrated in
495 plants, in addition to repellent effect against ants and antitumor, antimicrobial activities for
496 animals and microorganisms [44,46]. Dihydroactinidiolide has carbonyl group that can react with
497 nucleophilic structures in macromolecules, providing high potential reactivity to the molecules. It
498 is also showed cytotoxic effects against cancer cell lines Yun et al. [45]. The 7-Deoxyloganic
499 acid isomer was the other terpene, which is known, as intermediate in secoiridoid pathway in
500 plants.

501 In summary, the current study delivered deeper insights into JAPC evaluation originating from
502 fractionated green biomass of different Jerusalem artichoke clones. However future studies on

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503 anti-nutritional ingredients of JAPC as well as chemical composition of other fractions such as
504 brown juice and fiber along with economically calculation are needed to be address.

505 Conclusion

506 Considering the high amount of lingo-cellulose shoot biomass and inulin enriched tubers several
507 literatures suggest the utilization of Jerusalem artichoke in biorefinery context. Less information
508 is available about the green biomass utilization for green biorefinery purposes even Jerusalem
509 artichoke has repeatedly harvestable leafy shoot with valuable biochemical compounds. At least
510 one value-added product is necessary to produce in order to achieve cost-effective green bio-
511 refinery. Hence in the present research paper, we tried to highlight and deliver more information
512 about leaf protein concentrate (JAPC) generated from different Jerusalem artichoke clones using
513 biotechnological method. It can be harvested two times a year generating valuable quantities of
514 green aerial biomass under low inputs condition. Yield of JAPC was almost the same among
515 studied Jerusalem artichoke clones. Amino acids and fatty acid compositions, as value indicator
516 parameters, were similar in JAPC generated from Jerusalem artichoke clones. Present
517 biochemical analysis revealed that the JAPC is not only a good source of protein with favorable
518 amino acid composition but also it is repository of essential fatty acids, flavonoid and non-
519 flavonoid phytonutrients. We found that the quantity and/or quality of phytochemicals are
520 specific primarily for the Jerusalem artichoke species and for the technological way. Within the
521 species, slight difference can be revealed in the examined parameters between the clones. Results
522 of present work confirm that this underestimated plant can be directed not only towards tuber
523 production for inulin extraction, but the green biomass can also represent a value for JAPC
524 production under low inputs in green biorefineries.

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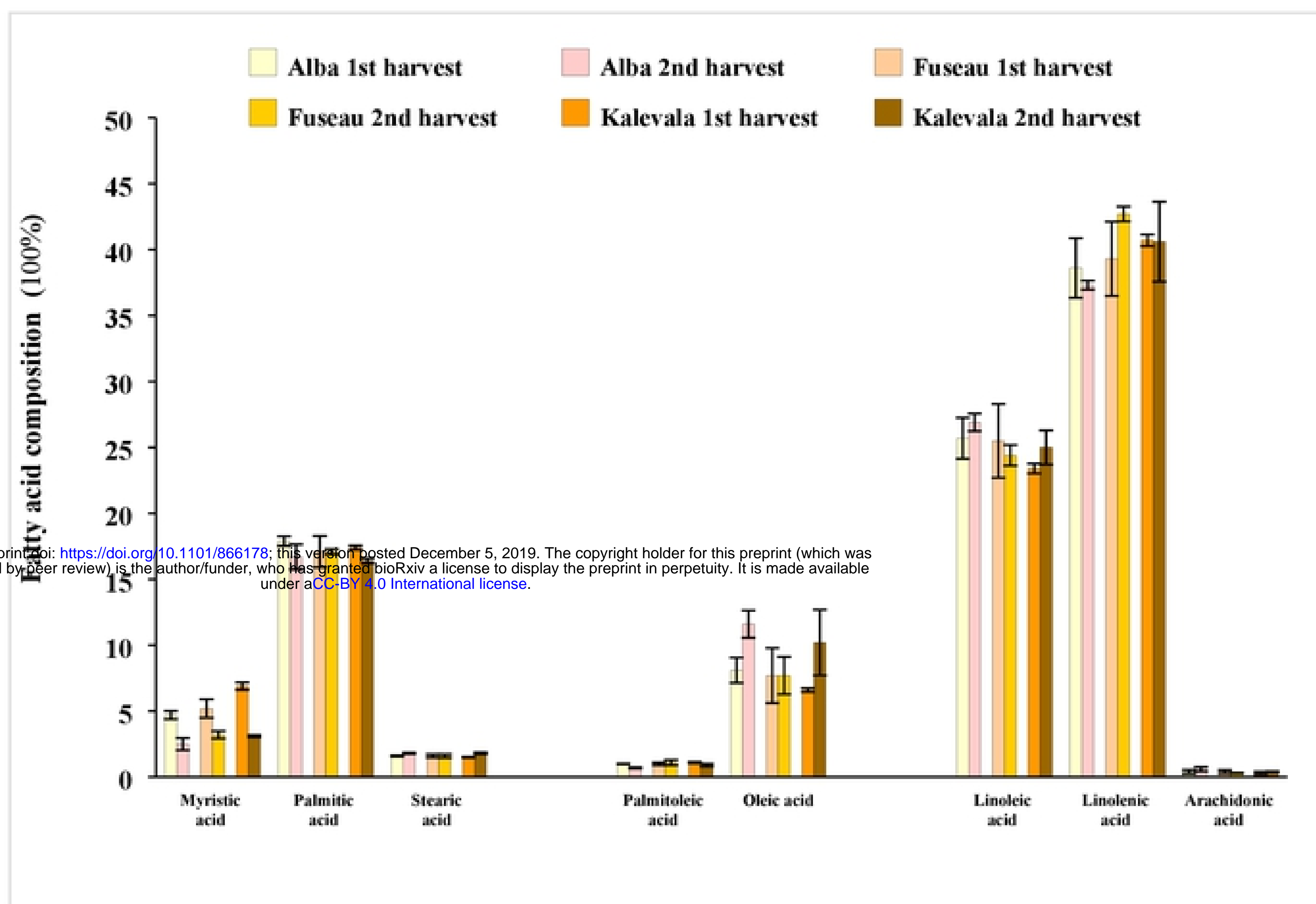

Fig 1. Fatty acid profile (100%) of Jerusalem artichoke leaf protein concentrate (JAPC) extracted from green biomass of different clones (Alba, Fuseau and Kalevala).

Figure 1

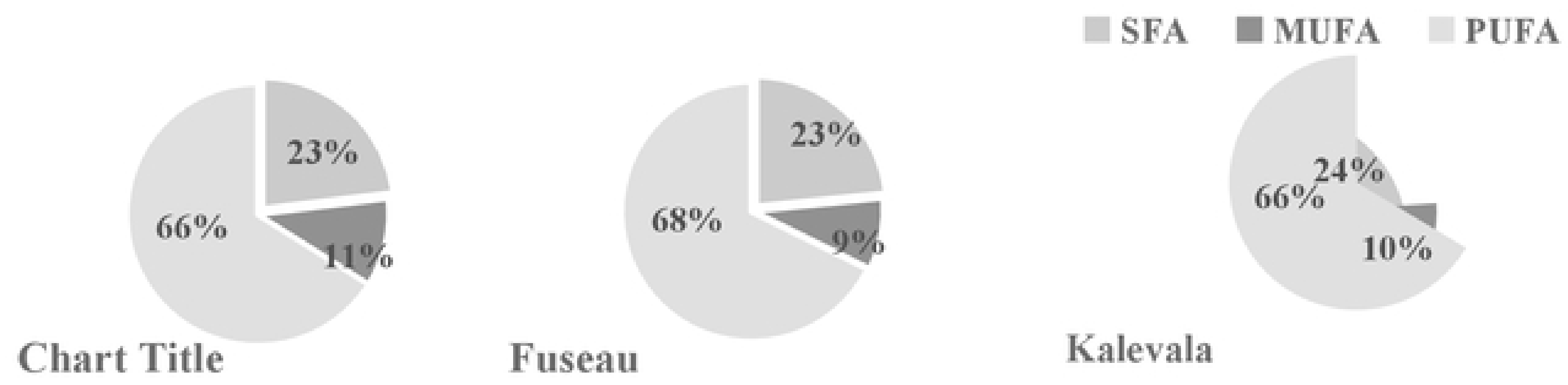

Fig 2. Distribution of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in Jerusalem artichoke leaf protein concentrate (JAPC) extracted from green biomass of different clones (Alba, Fuseau and Kalevala).

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Figure 2

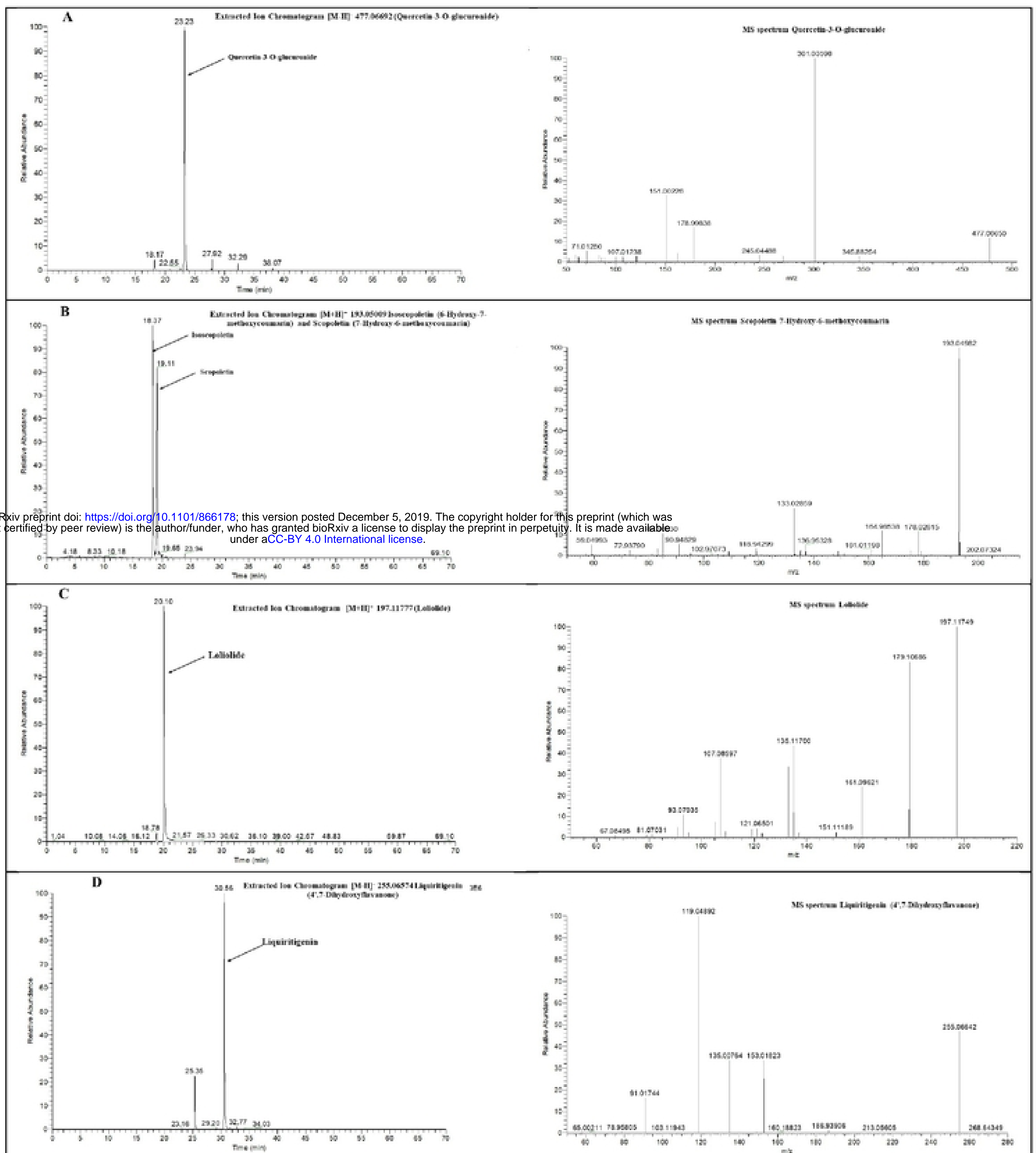

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Fig 3. Extracted Ion Chromatograms (XIC) and MS spectra of selected phytoconstituents from Jerusalem artichoke leaf protein concentrate: A: quercetin- 3-O-glucuronide; B: Scopoletin; C: Loliolide; D: Liquiritigenin

Figure 3

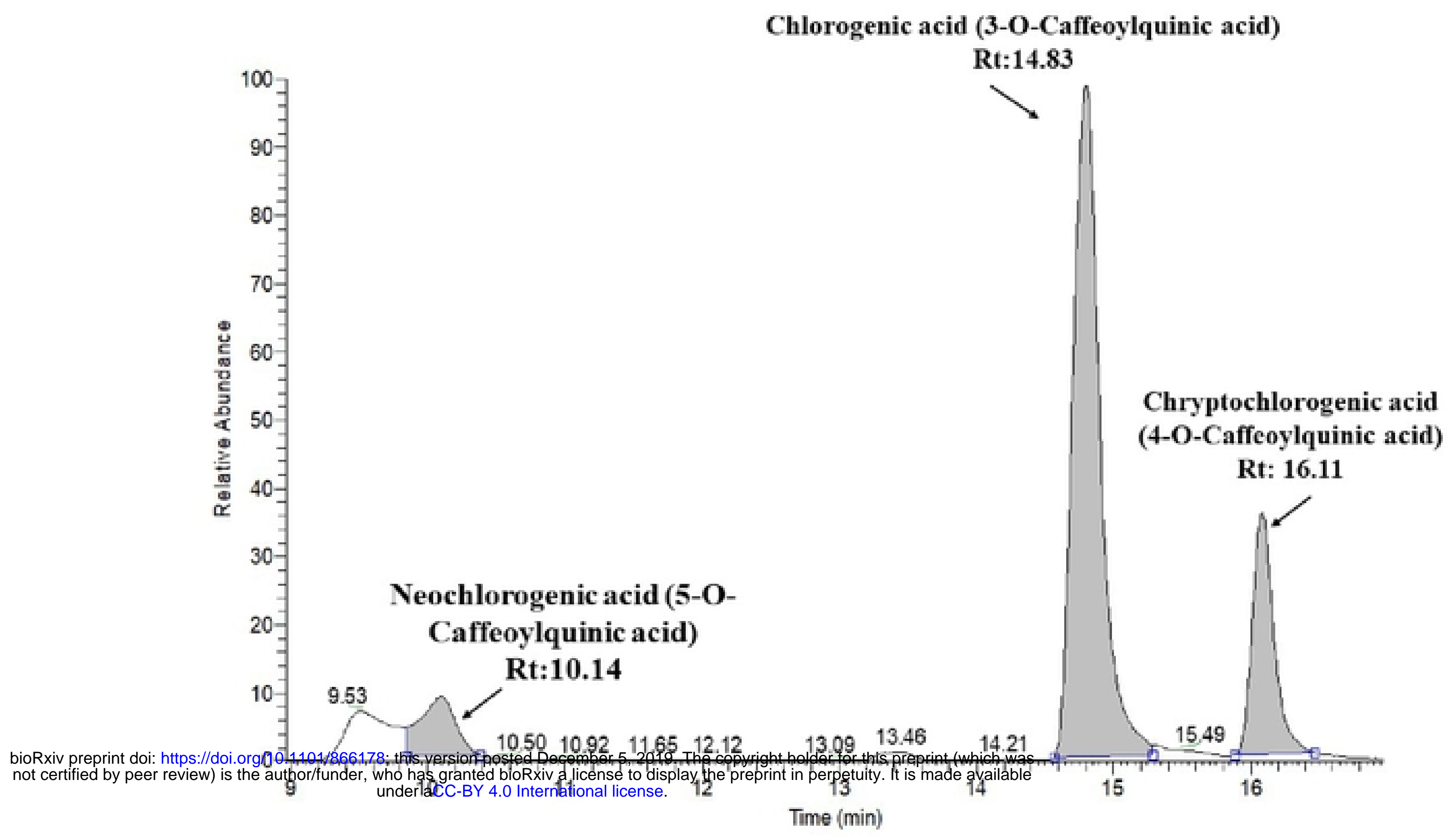

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Fig 4. Extracted Ion Chromatoram of chlorogenic acid isomers

Figure 4

Table 1. Aboveground fresh biomass, dry mass and total protein content of Jerusalem artichoke leaf protein concentrate (JAPC) isolated from green biomass of different clones

Clones	Fresh Biomass yield (kg m^{-2})		JAPC (g kg^{-1} fresh biomass) dry basis		Total Protein %	
	1st harvest	*2nd harvest*	*1st harvest*	*2nd harvest*	*1st harvest*	*2nd harvest*
Alba	5.0±0.43 a	1.8±0.22 b	31.9±2.7 a	30.4±3.2 a	35.3±0.8 a	31.6±0.8 b
Fuseau	5.2±0.28 a	2.6±0.19 ab	28.3±1.9 a	28.8±2.8 a	33.3±0.9 a	35.2±0.8 a
Kalevala	5.6±0.65 a	2.8±0.57 a	32.3±2.1 a	28.0±2.7 a	33.8±0.7 a	33.4±0.7 ab

Means followed by different letters in the same column show significant differences according to Duncan's test at $p < 0.05$.

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Table 1

Table 2. Amino acid profile (m/m%) of Jerusalem artichoke leaf protein concentrate (JAPC) extracted from green biomass of different clones

	Alba		**Fuseau**		**Kalevala**	
	1st harvest	*2nd harvest*	*1st harvest*	*2nd harvest*	*1st harvest*	*2nd harvest*
Lysine	2.32	2.35	2.19	2.54	2.25	2.46
Histidine	0.80	0.72	0.71	0.76	0.83	0.82
Isoleucine	1.72	1.72	1.64	1.86	1.77	1.78
Leucine	3.25	3.19	3.08	2.46	3.31	3.30
Phenylalanine	2.12	2.03	1.96	2.20	2.19	2.18
Methionine	0.87	0.82	0.84	0.95	0.79	0.77
Threonine	1.96	1.95	1.87	2.12	2.33	2.33
Valine	2.05	2.10	2.02	2.34	2.06	2.09
Alanine	2.36	2.32	2.20	2.47	2.35	2.34
Arginine	2.08	1.87	1.88	1.97	1.86	2.21
Aspartic acid	3.81	3.89	3.63	4.23	4.23	4.24
Cysteine	0.24	0.24	0.22	0.26	0.22	0.23
Glycine	2.04	1.99	1.93	2.14	2.13	2.14
Glutamic acid	4.29	4.38	4.14	4.74	4.82	4.79
Proline	1.92	2.04	1.82	2.18	2.20	2.19
Serine	1.74	1.77	1.67	1.89	1.90	1.93
Tyrosine	1.48	1.42	1.38	1.61	1.46	1.55
Ammonia	0.49	0.52	0.47	0.48	0.52	0.54

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Table 2

Table 3. Chemical composition of Jerusalem artichoke leaf protein concentrate (JAPC) extracted from green biomass

No.	Compound	Formula	Retention time	[M + H]+	[M - H]-	Fragmens 1	Fragmens 2	Fragmens 3	Fragmens 4	Fragmens 5
1	Lysine	C6H14N2O2	1,12	147,11336		130,0866	84,0814	67,0547	56,0503	
2	γ-Aminobutyric acid	C4H9NO2	1,25	104,07116		87,0446	86,0607	69,0342	58,0658	
3	Quinic acid	C7H12O6	1,27		191,05557	173,0447	171,0289	127,0388	93,0331	85,0280
4	Betaine (Trimethylglycine)	C5H11NO2	1,28	118,08681		59,0737	58,0659			
5	Proline	C5H9NO2	1,28	116,07116		70,0658	68,0500			
6	Glutamic acid	C5H9NO4	1,32	148,06099		130,0502	102,0555	84,0450	56,0502	
7	Glutamine	C5H10N2O3	1,32	147,07697		130,0502	102,0554	101,0716	84,0450	56,0502
8	Malic acid	C4H6O5	1,33		133,01370	115,0024	89,0230	87,0075	72,9916	71,0123
9	Nicotinic acid (Niacin)	C6H5NO2	1,51	124,03986		96,0450	80,0501	78,0347		
10	Citric acid	C6H8O7	1,73		191,01918	173,0082	129,0182	111,0075	87,0073	85,0280
11	Leucine and Isoleucine	C6H13NO2	1,84	132,10246		86,0970	69,0706			
12	Tyrosine	C9H11NO3	1,93	182,08172		165,0548	147,0443	136,0759	123,0444	119,0495
13	Phenylalanine	C9H11NO2	3,64	166,08681		149,0598	131,0495	120,0811	107,0496	103,0547
14	Tryptophan	C11H12N2O2	9,38	205,09771		188,0709	170,0602	159,0919	146,0602	118,0655
15	Neochlorogenic acid (5-O-Caffeoylquinic acid)	C16H18O9	10,14		353,08726	191,0557	179,0344	173,0448	135,0441	
16	Salicylic acid-2-O-glucoside	C13H16O8	13,56		299,07670	137,0234	113,0229	93,0331	85,0280	71,0123
17	Chlorogenic acid (3-O-Caffeoylquinic acid)	C16H18O9	14,83		353,08726	191,0556	179,0344	173,0443	161,0234	135,0441
18	Chryptochlorogenic acid (4-O-Caffeoylquinic acid)	C16H18O9	16,11		353,08726	191,0555	179,0344	173,0447	161,0232	135,0441
19	4-O-(4-Coumaroyl)quinic acid	C16H18O8	16,14		337,09235	191,0555	173,0447	163,0390	119,0489	93,0331
20	Vanillin	C8H8O3	16,22	153,05517		125,0600	111,0445	110,0366	93,0341	65,0393
21	5-O-(4-Coumaroyl)quinic acid	C16H18O8	17,38		337,09235	191,0556	173,0447	163,0391	119,0490	93,0332
22	Indole-3-acetic acid	C10H9NO2	17,98		174,05551	146,0601	144,0440	130,0651	128,0492	
23	4-O-(4-Coumaroyl)quinic acid cis isomer	C16H18O8	18,04		337,09235	191,0556	173,0447	163,0391	119,0489	93,0331

24	Isoscopoletin (6-Hydroxy-7-methoxycoumarin)	C10H8O4	18,33	193,05009		178,0264	165,0550	149,0598	137,0600	133,0287
25	5-O-Feruloylquinic acid	C17H20O9	18,42		367,10291	193,0503	191,0556	173,0447	134,0362	93,0331
26	Riboflavin	C17H20N4O6	19,03	377,14611		359,1352	243,0879	200,0824	172,0872	69,0342
27	Scopoletin (7-Hydroxy-6-methoxycoumarin)	C10H8O4	19,08	193,05009		178,0263	165,0546	149,0597	137,0601	133,0287
28	Azelaamic acid (9-Amino-9-oxononanoic acid)	C9H17NO3	19,21		186,11302	125,0959	97,0647			
29	6-Methylcoumarin	C10H8O2	19,44	161,06026		133,0651	115,0547	105,0704	91,0547	79,0549
30	5-O-(4-Coumaroyl)quinic acid cis isomer	C16H18O8	19,63		337,09235	191,0555	173,0446	163,0390	119,0491	93,0330
31	Indole-4-carbaldehyde	C9H7NO	19,67	146,06059		118,0655	117,0574	91,0548		
32	Fraxidin vagy Isofraxidin	C11H10O5	19,72		221,04500	206,0219	190,9983	163,0030		
33	Loliolide	C11H16O3	20,05	197,11777		179,1069	161,0962	135,1171	133,1015	107,0860
34	4-Hydroxy-3-methoxycinnamaldehyde (Coniferyl aldehyde)	C10H10O3	20,59	179,07082		161,0599	147,0442	133,0652	119,0495	55,0186
35	7-Deoxyloganic acid isomer	C16H24O9	22,36		359,13421	197,0815	153,0909	135,0805	109,0643	89,0230
36	Di-O-caffeoylquinic acid isomer 1	C25H24O12	22,61		515,11896	353,0884	191,0556	179,0342	173,0447	135,0441
37	Di-O-caffeoylquinic acid isomer 2	C25H24O12	22,77		515,11896	353,0884	191,0556	179,0342	173,0446	135,0440
38	Salvianolic acid derivative isomer 1	C27H22O12	22,80		537,10331	375,0705	201,0165	179,0343	161,0234	135,0440
39	Butein (2',3,4,4'-Tetrahydroxychalcone)	C15H12O5	23,00	273,07630		255,0656	227,0699	209,0602	163,0391	137,0235
40	Quercetin-3-O-glucuronide	C21H18O13	23,26		477,06692	301,0359	178,9980	163,0028	151,0026	121,0281
41	Isoquercitrin (Hirsutrin, Quercetin-3-O-glucoside)	C21H20O12	23,47		463,08765	301,0358	300,0283	271,0253	255,0300	
42	Chrysoeriol-O-glucoside	C22H22O11	23,87		461,10839	299,0560	298,0484	270,0537	255,0292	227,0346
43	Salvianolic acid derivative isomer 2	C27H22O12	24,60		537,10331	375,0705	201,0166	179,0343	161,0236	135,0440

44	Di-O-caffeoylquinic acid isomer 3	C25H24O12	24,62		515,11896	353,0884	191,0557	179,0342	173,0447	135,0440
45	Azelaic acid	C9H16O4	25,05		187,09704	169,0863	143,1070	125,0959	123,0803	
46	Kaempferol-3-O-glucuronide	C21H18O12	25,18		461,07200	285,0410	229,0505	113,0231		
47	Apigenin-O-malonylglucoside	C24H22O13	25,21		517,09822	473,1116	269,0461	268,0376		
48	Astragalin (Kaempferol-3-O-glucoside)	C21H20O11	25,26		447,09274	285,0410	284,0331	255,0302	227,0350	
49	Isorhamnetin-3-O-glucoside	C22H22O12	25,48		477,10330	315,0524	314,0437	285,0406	271,0248	243,0292
50	Kukulkanin B (3'-Methoxy-2',4,4'-methoxychalcone)	C16H14O5	25,50	287,09195		269,0810	241,0864	177,0548	145,0286	137,0235
51	Isorhamnetin-3-O-glucuronide	C22H20O13	25,70		491,08257	315,0517	300,0275	271,0249		
52	Dihydroactinidiolide	C11H16O2	27,16	181,12286		163,1119	145,1014	135,1171	121,1015	107,0860
53	Dimethoxy-tetrahydroxyflavone	C17H14O8	28,38		345,06105	330,0386	315,0153	287,0204	215,0347	178,9978
54	Dihydroxy-methoxyflavone	C16H12O5	29,89		283,06065	268,0381	267,0305	240,0427	239,0350	211,0396
55	Dimethoxy-trihydroxyflavone isomer 1	C17H14O7	30,09		329,06613	314,0439	299,0197	283,0869	271,0247	255,0913
56	Trihydroxy-trimethoxyflavone	C18H16O8	30,36		359,07670	344,0541	329,0307	314,0075	301,0358	286,0129
57	Dimethoxy-trihydroxyflavone isomer 2	C17H14O7	30,38		329,06613	314,0439	299,0201	283,0871	271,0252	253,0763
58	Liquiritigenin (4',7-Dihydroxyflavanone)	C15H12O4	30,56		255,06574	153,0183	135,0077	119,0489	91,0175	
59	Hymenoxin (5,7,Dihydroxy-3',4',6,8-tetramethoxyflavone)	C19H18O8	32,11	375,10800		360,0840	345,0606	342,0736	330,0367	317,0659
60	Epiafzelechin trimethyl ether	C18H20O5	33,32	317,13890		167,0704	163,0755	155,0705	137,0598	121,0651
61	Nevadensin (5,7-Dihydroxy-4',6,8-trimethoxyflavone)	C18H16O7	33,91	345,09743		330,0736	315,0501	312,0631	287,0554	