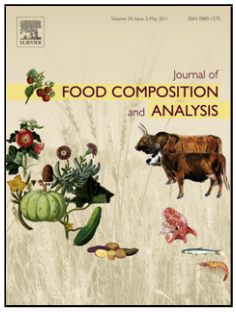

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Authors: Arianna Ricci, Giuseppina P. Parpinello, Aline S. Palma, Nemanja Teslić, Claudio Brilli, Antonio Pizzi, Andrea Versari

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Original research paper

Analytical profiling of food-grade extracts from grape (*Vitis vinifera sp*) seeds and skins, green tea (*Camellia Sinensis*) leaves and Limousin oak (*Quercus robur*) heartwood using MALDI-TOF-MS, ICP-MS and spectrophotometric methods

Arianna Ricci[a], Giuseppina P. Parpinello[a*], Aline S. Palma[a,b], Nemanja Teslić[a], Claudio Brilli[c], Antonio Pizzi[d,e], Andrea Versari[a]

[a] Department of Agricultural and Food Sciences, University of Bologna, Piazza Goidanich 60, Cesena (FC) 47521, Italy

[b]CAPES Foundation, Ministry of Education of Brazil, Brasilia – DF 70040-020, Brazil.

[c]RUFFINO s.r.l. –Constellation Brands Company Analytical Laboratory, P. le Ruffino, 1, Pontassieve (FI) 50065, Italy

[d] LERMAB, University of Lorraine, 27 rue du Merle Blanc, BP 1041, 88051 Epinal, France

[e] Department of Physics, King Abdulaziz University, Jeddah, Saudi Arabia

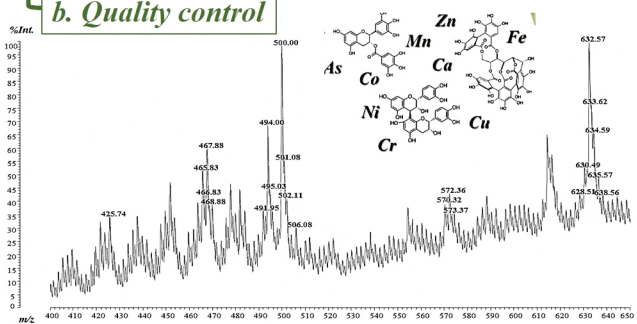
***Corresponding author: giusi.parpinello@unibo.it**

Graphical abstract

Natural extracts:

- **a. Sources selection**

- **b. Quality control**



- **c. Exploitation in the food industry and animal nutrition**

Highlights

- MALDI-TOF-MS and UV-Vis informed on food-grade tannins composition and authenticity.
- The extract from green tea showed a high content of flavonol glycosides.
- The condensed tannins were discriminated on the basis of the galloylation patterns.
- The extract from Limousin oak was rich in castalagin and polygalloylglucosides.
- ICP-MS highlighted the elemental composition of tannin additives.

Abstract

Tannins are food additives widely used due to their antioxidant and antimicrobial activities, and flavouring properties. Nevertheless, the information provided by the manufacturers are often generic, and data on the presence of specific phenolic compounds and potentially toxic elements are needed. In this work a selection of food-grade plant extracts – also called 'commercial tannins' – from different botanical sources: *Vitis vinifera sp*, *Camellia Sinensis*, *Quercus robur*, were profiled using UV-visible spectrophotometry, matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS), and inductively coupled plasma-mass spectrometry (ICP-MS). The combined analytical approach was suitable for quality control of the polyphenolic fraction to highlight authenticity markers (e.g. galloylated flavonoids, glycosides, ellagitannins), and to ascertain the content of toxic elements (Cu, Sr, As, Co, Cr, Fe, Zn, Li, Ba and Pb) respect to their legal and/or recommended limits.

Keywords: Food analysis, food composition, procyanidins, ellagitannins, tannins authenticity, tannins quality, metals toxicity.

Introduction

There is an increasing interest in the exploitation of phytochemicals from agri-food by-products and wastes. In this perspective, tannins – secondary phenolic metabolites produced by plants – have gained increasing interest for their exploitation in the food industry, due to their application in human and animal nutrition as natural antioxidants (Arogba, 2000; Chung, Wong, Wei, Huang, & Lin, 1998) and feed flavourings (EFSA FEEDAP Panel, 2014).

Commercial tannins are natural polyphenolic compounds occurring in plant woods, fruits, seeds, and extracted from different plant tissues. Few selected species are employed in the food technologies, due to their high content in bioactive compounds which reflects their technological performances. Procyanidins are condensed molecular structures composed of flavan-3-ol monomeric units; the main natural sources are constituted by grape seed, skin and leaves, mimosa bark, Quebracho wood, and tea leaves. Hydrolysable tannins consists of glycosylated units of gallic and ellagic acid monomers and polymers; the oak, chestnut and gallnut woods provide primary sources of hydrolysable tannins for the food industry (Versari, du Toit, & Parpinello, 2013).

Tannins consist of a complex matrix including polymerized fraction and additional components such as phenolic monomers, oligomeric fractions, phenolic aldehydes, stilbenes, sugars and glycosylated compounds, among others (Versari, du Toit, & Parpinello, 2013). The properties of tannins are strongly dependent on the composition of specific classes of bioactive compounds occurring as a consequence of the extraction process, and on the lack of contaminants (Vivas, Nonier, de Gaulejac, Absalon, Bertrand, & Mirabel, 2004). In particular, the phenolic compounds can remove free radicals and chelate transition metals and proteins, improving quality and stability of food and beverages (Haslam, 1998). However, tannins could induce astringent, antinutritional and toxic effects (Avallone, Plessi, Baraldi, & Monzani, 1997; Makkar, 2003). Therefore their analytical characterization is a suitable tool for the valorisation of plant extracts (e.g. tea leaves) and for their

exploitation as sources of food supplements for human nutrition and animal feed. In particular, the bioaccessibility and fermentability of tannins by colonic microflora is highly affected by their degree of polymerisation (Serrano, Puupponen-Pimiä, Dauer, Aura, & Saura-Calixto, 2009).

The use of tannins in the food industry is regulated by Communitarian and International legislations (FDA- Code of Federal Regulations Title 21; Directive 2012/12/EU of the European Parliament and of the Council, amending Council Directive 2001/112/EC; Beer Judge Certification Program (BJCP) Style Guidelines 2015; Resolution OIV-OENO 554-2015, among others) but limited recommendations are provided for the authentication and typification of raw materials. The composition of the monomeric polyphenolic fraction and building blocks of oligomers are specific for each botanical class, and provide information on the effective origin of the extracts. The main markers of quality and authenticity of commercial tannins are related to the content of condensed and hydrolysate structures, also including (i) the degree of galloylation of polygalloylglucose chains and flavanol monomers, (ii) the glycosylation patterns derived from degradation of plant tissues, and (iii) their molecular arrangement following extraction (Amakura, Yoshimura, Sugimoto, Yamazaki, & Yoshida, 2009; Nonier, Vivas, De Gaulejac, Absalon, Vitry, & Fouquet, 2005)

One of the most beneficial groups of polyphenolic compounds obtained by natural extracts is constituted by proanthocyanidins, with procyanidins as the most prominent subclass; mixtures of procyanidolic oligomers naturally occur in fruits, with red fruits and grapefruit as the main sources, and tea leaves. The related extracts have technological and nutritional impact, being exploited both in the beverages industry and as food supplement. The grape extracts are commonly composed of flavonoid monomers and procyanidin oligomers and polymers extracted from *Vitis vinifera sp.* grape seeds and skin; among them, a large amount of flavan-3-ol monomers esterified with gallic acid units has been observed in the grape seed procyanidins (Krueger, Dopke, Treichel, Folts, & Reed, 2000), resulting in an enhanced bioactivity and a stronger sensory impact when compared to the skin extract.

Due to the steric hindrance produced by esterification of building monomers, the degree of polymerisation of procyanidins could reach a maximum of 16 units; the occurrence of higher polymers may derive from oxidative condensation of oligomers following extraction (Prieur, Rigaud, Cheynier, & Moutounet, 1994). The grape skin extract is characterised by a higher degree of polymerisation, related to the low degree of esterification of flavan-3-ol monomeric units. Both skin and seed extracts from grape are effective antioxidants and can be added to foods and beverages to retard deterioration.

Green tea (*Camellia sinensis*) leaves are recognized as a major source of galloflavonoid and flavonoid gallates, with a prevalence of (−)-epigallocatechin gallates (Graham, 1992; Yoshizawa, Horiuchi, Fujiki, Yoshida, Okuda, & Sugimura, 1987); the peculiar composition makes the extract a suitable candidate as a protection from oxidative stress (Frazier, Deaville, Green, Stringano, Willoughby, Plant, et al., 2010; Perumalla & Hettiarachchy, 2011). Nevertheless, the ability of galloylated structure to interact with proteins is also responsible for the astringent perception induced by gallic acid-based compounds (Harbertson, Parpinello, Heymann, & Downey, 2012; Obreque-Slier, López-Solís, Peña-Neira, & Zamora-Marín, 2010); the use of both grape seed extract and green tea tannins would require a detailed investigation on the chemical composition of the phenolic fraction and on the sensory impact for their addition.

The water-soluble hydrolysable tannins contained in European oak (*Quercus* species) heartwood have been historically exploited for cooperage, and they are recognized as providing beneficial stabilising and flavouring effects. Oak wood is mainly used for the conservation of fine beverages: aging wines, spirits, and balsamic vinegars are traditionally stored in barrels, where the continuous contact with wood naturally extracts and releases ellagitannins in solution; ellagitannins contained in the oak extracts increase the antioxidant activity, due to the high content in gallic and ellagic acids-based compounds (Landete, 2011). Several scientific surveys have explored the differences between European and American *Quercus* species used for cooperage, to determine the most suitable

formulation for food industry needs. Among them, the work of Chatonnet et al. (1998) has highlighted the supremacy of oak obtained from Limousin forests in relation to its content in water-soluble precursors (Chatonnet & Dubourdieu, 1998). Limousin oak forests are located in the southwest area of France, with a prevalence of *Q. robur* trees; this species has a high content in ellagitannins, vanillin and phenolic aldehydes when compared to *Q. petraea* and *Q. alba* oak species (Chatonnet & Dubourdieu, 1998).

Since there is no regulation constraining manufacturers to provide a detailed composition of the extracts or the methods of extraction used, information on adulteration and possible contamination is not available to the consumer, and the purity and the healthiness of commercial tannins can be questionable. Monitoring the composition of food additives is a key step to guarantee consumer safety, through the identification of markers (both qualitative and quantitative) which inform on their effective content in bioactive compounds, their authenticity and non-toxicity (Li & Vederas, 2009; Pennington, 2002). Moreover, a correct balance in macroelements (Ca, K, Mg, Zn, among others) could enhance the nutritional value of this food additive (Sulaiman, Yusoff, Eldeen, Seow, Sajak, & Ooi, 2011; Vin, Papadopoulos, Cubadda, Aureli, Basegmez, D'Amato, & Lucarini, 2014).

Due to the large variety of commercial botanical extracts used in the food industry, there is a need to explore both targeted and non-targeted approaches to assess the quality/authenticity of commercial tannin formulations. In this work, the compositional parameters of four tannins employed for human consumption and selected to represent commercially available extracts from: grape seed, grape skin, green tea leaves, and Limousin oak heartwood, were investigated, and quality and authenticity markers were identified. MALDI-TOF MS, ICP-MS and spectrophotometric assays were used for qualitative and quantitative analysis; in particular, the elemental and molecular composition, along with the polyphenolic content and antiradical activity were assessed for these commercial food additives, and briefly discussed.

2. Experimental

2.1. Chemicals

Solvents, L-tartaric acid (≈ 100%) pure ethanol (>99%) to prepare tannin solutions, and HNO_3 and H_2O_2 reagent grade used for ICP-MS analysis were supplied by Merck (Darmstadt, Germany). Bovine serum albumin (BSA), anhydrous $FeCl_3$ and 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) for spectrophotometric determinations were purchased by Sigma Aldrich (St. Louis, MO). Phosphorus red reference standard (99,999%) used for MALDI-TOF analyses was purchased from Acros Organics (Fair Lawn, NJ).

2.2. Samples preparation

Four food-grade commercial tannins from different botanical sources: grape seed (SEP) and skin (SKP) proanthocyanidins, green tea procyanidins (GTP), and Limousin oak ellagitannin (LOE), were purchased from the Enologica Vason company (Enologica Vason S.p.A., Verona, Italy) as lyophilized powders and stored at room temperature; any purification treatment was carried out on commercial samples before analyses. According to Harbertson et al. (2002) 1 g/L of tannin powders were dissolved in hydro-alcoholic solution (12% (*v/v*) ethanol in distilled water, with addition of L-0.033 M tartaric acid and 1 M NaOH to reach pH 3.6) to obtain a 'stock solutions' of each sample; then, stock solutions were properly diluted in distilled water for spectrophotometric assays.

For MALDI-TOF experiments, powder samples were dissolved in acetone/water 50/50% (*v/v*) at a concentration of 5 mg/mL, and then the solutions were mixed (1:1, *v/v*) with a standard solution of 2,5-dihydroxybenzoic acid at 10 mg/mL dissolved in the same solvent. Then, 1.5 µL of the mixed solution were spotted on a 384-well MALDI-TOF plate, followed by evaporation of the solvent at ambient temperature before analysis.

For ICP-MS analysis the stock solutions (5 mL) were added with 3 mL of HNO_3 and 2 mL of H_2O_2 reagent grade, then heated to 130 °C (digester DigiBlock ED365; LabTech, Hopkinton, MA) for 3 hours; subsequently the mixture was diluted with 25 mL of ultrapure H_2O before analysis.

2.3. Determination of total polyphenols, tannin fraction and *in vitro* antioxidant capacity

Total (iron reactive) polyphenols and tannins were quantified using the method of Harbertson et al. (Harbertson, Kennedy, & Adams, 2002) which is based (i) on the ability of protein (i.e. bovine serum albumin, BSA) to precipitate tannins, and (ii) on the reactivity of ferric chloride with phenolic compounds that possess *ortho*-dihydroxyl groups, as previously described (Versari, Parpinello, & Mattioli, 2007). This method is particularly suitable for commercial tannins analysis as it estimates the degree of purity and the amount of iron-reactive polyphenolics, which play a critical role in redox systems.

The *in vitro* antioxidant capacity of tannin samples was determined using the DPPH• radical scavenging method (Brand-Williams, Cuvelier, & Berset, 1995) that evaluate of the decay of radical absorbance at 517 nm; results were expressed as Trolox equivalents (mmol TE/L).

All spectrophotometric determinations were performed using a Shimadzu UV Mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan).

2.4. MALDI–TOF mass spectrometry

MALDI-TOF spectra were recorded using a Kratos compact MALDI Axima Performance TOF 2 instrument (Shimadzu Biotech, Manchester, UK), equipped with a nitrogen laser (337 nm), an ion gate for the selection of precursor ions, and a collision cell, according to Lagel et al. (2014). The windows for separation of precursor ions were approximately 4 Da. Argon has been used as the collision gas. All data were obtained in positive ion linear mode applying the accumulation of 441 scans per spectrum. Linear negative mode was used for the investigation of hydrolysable and

glycosylated molecular patterns which characterise the Limousin oak extract. The calibration of the linear modes was done using phosphorus red pigment as a reference over a mass range up to 2500 Da. NaCl was added in the sampling wells as the salt to enhance ion formation previous deposition of samples. The MALDI-TOF target was then analysed to give the resulting spectra, using a raster analysis over the target; Maldi-MS software was used for data treatment (Shimadzu Biotech, Manchester, UK).

2.5. ICP-MS analyses

Metals were analyzed using an Agilent ICP-MS equipped with a 7700 Q Apex + Spiro TMD (Agilent Technology, Santa Clara, CA); the standard nebulizer for sample introduction was replaced with a desolvation system. The APEX Q atomizes the sample into a cyclonic chamber heated to 140 °C and subsequently cooled, thus inducing the removal of most of the aqueous component of the sample. After condensation; this process enables concentration of the sample by about 10 times. The Spiro TMD (Teflon membrane desolvator) is composed of a spiral interfaced with a Teflon membrane heated at 100 °C that further removes the water vapor from the sample aerosol stream, enabling only the dry component to reach the plasma. The acid digestion step and the combined use of APEX Q and Spiro TMD technologies enable the simultaneous determination of macro, micro and trace elements, and it is routinely used as an internal laboratory protocol for elemental profiling; nevertheless, in this work only the first two categories showed significant concentrations and are accordingly discussed in the Results and Discussion section. The instrument is also equipped with a collision chamber sparged with He for the removal of interferences, such as oxides (below 0.1%); analyses were performed with and without He stream, to avoid overestimation due to occasional adducts formed.

Internal standards Ge and Tl were added through a tee into the Apex, before the introduction of the sample and used for quantitation.

2.6. Statistical analysis

Microsoft Excel was used for data entry, and statistical analysis was performed with Unscrambler X.1 (Camo ASA, Oslo, Norway). All analyses were performed in triplicate and the results expressed as mean ± standard deviation (SD). The statistically significant level was considered at $\alpha = 0.05$.

3. Results and Discussion

3.1. Total (iron reactive) polyphenols, tannin fraction and *in vitro* antioxidant capacity

The total polyphenol content, tannin fraction and the *in vitro* antioxidant capacity of the four commercial extracts (**Table 1**) was carried out to set-up a rapid screening approach to provide both qualitative and quantitative information useful for quality control of commercial products. Samples were ranked in terms of total polyphenols content as follows: SKP > LOE > GTP > SEP.

The SKP extract peaked in the total polyphenol content (2.83 mM CE), of which 42.9% is the polymeric tannin fraction (1.23 mM CE). The seed procyanidin (SEP) was lowest in total polyphenols (1.62 mM CE), most of which (93%) comprised the polymerised fraction (flavonoid oligomers and polymers). The SKP/SEP ratio of proanthocyanidins estimated in this work (value = 1.3) is almost doubled when compared to the value of 0.55 obtained by Vivas et al. (2004) when studying similar commercial formulations. This result emphasised the high variability of bioactive compounds in commercial extracts, which is affected by several factors, including the extraction process (e.g., time, temperature, solvent, etc.) and the raw material (e.g., grape variety and maturity). Although the tannin fraction (% of the total polyphenol content) defines the effectiveness of the extraction process and influences the technological potential of the extracts for industrial applications (Kallithraka, Garcia-Viguera, Bridle, & Bakker, 1995), the monomeric fraction contributes to the antioxidant properties, and constitutes an important parameter to be monitored. The green tea extract showed a high content in polyphenolic compounds (2.11 mM CE), part of which was tannins

(47.6%), and the highest antioxidant capacity as radical scavenging (0.42 mM TE). The green tea leaves are rich in flavonoid-based monomers with a high degree of galloylation (Perumalla & Hettiarachchy, 2011), and the effectiveness of the galloylation patterns in the radical scavenging activity was also observed in the SEP sample (0.26 mM TE) compared to SKP (0.24 mM TE). The enhanced antioxidant capacity of Limousin oak extract (0.39 mM TE) compared to the grape extracts can be explained by the high content in total polyphenols (2.34 mM CE), with a great content in hydrolysable tannins (60.9%) that provides an effective protection against oxidation.

In general, samples showed a variable content in non-phenolic compounds (SEP > GTP > LOE > SKP), which was attributable to the presence of degradation by-products following extraction or addition of stabilising additives during processing, i.e. arabic gum powder, proteinaceous material, cellulose (Romani, Ieri, Turchetti, Mulinacci, Vincieri, & Buzzini, 2006).

3.2. Targeted analysis by MALDI-TOF mass spectrometry

3.2.1. Seed proanthocyanidin (SEP)

The MALDI-TOF profile of SEP sample highlighted the galloylation patterns that are a valuable marker of extracts from grape seeds and skin (Souquet, Cheynier, Brossaud, & Moutounet, 1996), the latter with high polymerization index and less astringent sensation (Vidal, Francis, Guyot, Marnet, Kwiatkowski, Gawel, et al., 2003). The SEP showed monomers from fragmentation patterns of flavonoid compounds (231 Da), whereas the 271 Da peak was attributable to fisetinidin (or catechin with loss in –OH), while the catechin was present in protonated form (291 Da). The occurrence of (–)-gallocatechin/ (–)-epigallocatechin compounds was represented by the peak at 303 Da, together with typical fragments 258–220 Da [MS^2 (*m/z*)]. The 152 Da fragment was related to the presence of galloyl units released during fragmentation. The major flavonoid monomeric units were found to be (+)-catechin/(–)-epicatechin (mass increment: 289 Da). However the building block of the first series of polymeric structures was represented by a fisitinedin-fisetinidin dimer (540 Da),

increasing its degree of polymerisation with catechins as repeating unit (**figure 1**): 827 Da (trimer); 1125 Da (tetramer); 1416 Da (pentamer); 1703 Da (hexamer); 1992 Da (heptamer); 2278 Da (octamer). Although the MALDI-TOF analysis was not able to discriminate between stereoisomers, due to the steric hindrance provided by flavanol units, we hypothesized a (–)-epicatechin-like structure as a building block for grape seed procyanidins, with (+)-catechin as a possible terminal unit (Vivas, Nonier, & Gaulejac, 2004). The second series of polymeric structures was characterised by the presence of galloylated compounds with a variable number of galloyl units, as summarized in **Table 2**. Flavonoid compounds were also found in the grape seed extract, as sodium adducts (309 Da, kaempferol; 324 Da, quercetin); on the basis of their molecular weight, the 463 peak was attributed to the quercetin-3-glycoside monomer, and the 609 Da peak was related to a quercetin-3-rutinoside (rutin) – like structure.

3.2.2. Skin proanthocyanidin (SKP)

The MALDI-TOF spectra of the grape skin proanthocyanidin (SKP) showed (+)-catechin/(–)-epicatechin (peak 283 Da) and gallocatechin/epigallocatechin (peak 303 Da with additional fragments 250 and 226 Da) as the main units, in agreement with the literature (Souquet, Cheynier, Brossaud, & Moutounet, 1996). Noteworthy, the peaks at 166/169 Da (gallic acid), 436 Da ((–)-epicatechin-3-gallate) and 462–464 Da ((−)-epicatechin-3-gallate sodium adduct) were markers for the presence of galloylated units. Catechin gallates are generally recognized as the main constituents of grape seed extracts (Vivas, Nonier, & Gaulejac, 2004; Yang & Chien, 2000), with a variable degree of galloylation in grape skins, mainly featured as (–)-epicatechin-3-*O*-gallate structures (Souquet, Cheynier, Brossaud, & Moutounet, 1996). The 463 Da peak could be alternatively assigned to the occurrence of quercetin-3-glycoside (isoquercetin), although not associated with complimentary fragmentation patterns. The only confirmation for this attribution derived from the

presence of the peak at 617 Da, as a possible isoquercetin gallate dimer. The 605 Da peak was referred to as quercetin-3-rutinoside (rutin) with loss in protons.

The composition of procyanidins for the SKP sample is listed in **Table 3**. It is mainly composed of three building blocks: catechin dimers (procyanidins B), catechin-gallocatechin dimers, fisetinidin dimers; among these three combinations, the main repeat unit consists of catechins (+288 Da, **Figure 2**).

3.2.3. Green tea procyanidins (GTP)

The green tea leaves were characterized by prodelphinidin compounds and flavonol-glycosides (**Table 4)**. According to the procyanidins composition, two series were detected in the MALDI-TOF spectra: the first series was characterised by procyanidin dimers, trimers and tetramers (mostly prodelphinidin), with various combinations of monomeric units. The 467 Da peak was attributed to catechin gallate or robinetinidin gallate, whereas the 628 Da was tentatively attributed to a catechin digallate protonated adduct that might represent a marker for authenticity of the extract. Despite the presence of the 574 Da peak – attributed to a procyanidins B-like structure – the oligomers series was mainly characterised by gallate compounds: 735 Da (catechin gallate-fisetinidin, dimer); 889 Da (catechin gallate-fisetinidin gallate, dimer); 903 Da (catechin gallate-catechin gallate, dimer); 1775 Da (3 catechin gallates units-fisetinidin gallate, tetramer). The 1978 Da and 2419 Da were assigned to the same series and were composed of catechin gallate unit linked to higher oligomers.

Remarkably, the mass region of ion peaks of the second series (951-1112-1274-1436-1602-1765-1927-2088-2247-2406), with occasional loss in protonated adducts, was observed with a peak-to-peak mass difference of 162 Da, consistent with the repeating unit of a hexose structure, possibly glucose (**figure 3**). This finding suggested the presence of glycosylated chains, either derived from degradation products of the foliar tissue or related to rearrangement of glucose release during extraction, since flavonoids in green tea are predominantly present as glycosides rather than non-

glycosylated forms (Wang & Helliwell, 2001). As an alternative hypothesis, the use of a sugar-based chain as a stabilising agent was formulated (although not labelled by suppliers).

3.2.4. Limousin oak ellagitannin (LOE)

The Limousin oak heartwood extract was rich in distinctive compounds, such as valoneic acid, polygalloylglucose structures, aldehyde derivatives and ellagitannins. The occurrence of a hydrolysable tannin is unambiguously confirmed by the presence of fragmentation patterns related to the polygalloylglucose structures: the 127 Da peak is related to cleavage mechanisms involving the glucose ring; the 152 Da peak is attributed to galloyl moieties (coupled to the 166 Da peak, due to the presence of gallic acid molecules). Moreover, ellagic acid (305 Da) and hexahydroxydiphenic acid (HHDP, 343 Da) monomers were detected in the extract, which are typically found in ellagitannin-based extracts. Several patterns were attributed to the fragmentation of polygalloyl compounds and sugar chains, which are likely to derive from oak wood following extraction; the basic unit for this series was a glucose dimer (glucose + glucose fragment, 219 Da; sodium adduct: 248 Da). **Table 5** provides a list of the fragmentation patterns produced by the Limousin oak tannin, including polygalloyl glucoses: the gallic acid multiprotonated residues linked as esters, through carboxylic acid moiety, to sugar or gallic acid molecules (**Figure 4**). Vescalagin/castalagin (934 Da) were detected as the main constituents of the extract, as confirmed by the 593, 623 and 609 Da peaks attributed to castalin/vescalin fragments (most likely castalin with loss in –OH moieties, and castalagin molecule with loss in an ellagic acid function and a water molecule, respectively). The occurrence of these compounds in the *Q. robur* extract is consistent with the literature (Nonier, Vivas, De Gaulejac, Absalon, Vitry, & Fouquet, 2005). The sodium adduct of roburin A/roburin D dimers (1873 Da peak) can be considered a marker for the *Quercus* wood extract.

3.3. Elemental profiling

Besides four macroelements (^{24}Mg, ^{39}K, ^{44}Ca and ^{55}Mn), twelve trace isotopes (^{7}Li, ^{27}Al, ^{52}Cr, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{88}Sr, ^{75}As, ^{138}Ba and ^{207}Pb) were investigated as possible contaminants derived from the botanical source or processing. Overall, the concentrations of macroelements ranged from 0.08 to 0.5 ppm for Mg, and from 0.2 to 0.4 ppm for Ca. The microelements ranged between 0.001–0.01 ppm for Mn, up to 0.99 ppm for Zn, and around 0.0001 ppm for Co (**Table 6**). Iron and copper were generally present in samples at very low levels (GTP sample: 0.06 ppm ^{56}Fe and 0.04 ppm ^{63}Cu, respectively) which is suitable for beverage application. Cu, Fe and Mn are important catalysts of oxidation of organic substrates (Danilewicz, 2007) and they form stable complexes with polyphenols, affecting the aroma, taste and color of many beverages, including wine (Waterhouse & Laurie, 2006) and beer (Bamforth, 2011).

As, Ni, Cu, and Ba, with respective security limits of 0.01, 0.07, 2.00, and 0.70 ppm recommended by WHO (2004), were far below the legal limits for all samples assayed. Although Cr, Li, Al and Sr are not included in the WHO guidelines, their concentrations lay within the ppb level in the four samples. In particular aluminum, which is likely to induce clouding and decreasing wine stability at increasing pH values (Mrak, Cash, & Caudron, 1937), was below the limit of quantitation (in the order of ppt) for all samples investigated. The content of Cr, which is crucial in foods due to its toxicity, ranged between 8.2–10.7 ppb, which is far below the ppm levels previously published for tannin extracts (Zmozinski, Pretto, Borgesa & Vale, 2015, Mehra, Lynch, Saikat, & Chan, 2013).

The Pb levels exceeded the limit set up by WHO for drinking waters (**Table 6**); the occurrence of the same Pb level in all samples suggested that a possible source of contamination occurred during processing rather than from the botanical sources themselves. Besides the WHO recommendations, several Community regulations have focused on exposure of humans to lead through food, and the SCF suggested a PTWI (provisional tolerable weekly intake) of 25 μg/kg bw (Reports of the Scientific Committee for Food, 32nd series, 19 June 1992); in more recent times, the Scientific Committee has stressed the hazard related to metal contaminant intake with a directive that

encourage the minimisation of lead levels in food (Reports on tasks for scientific co-operation, task 3.2.11: Assessment of dietary exposure to arsenic, cadmium, lead and mercury of the population of the EU Member States, Directive 93/5/CEE, 2004).

It has to be noticed that the maximum concentration of Pb allowed in wine is 0.3 mg/L according to the International Organization of Vine and Wine (OIV); accordingly, the lead level in the extracts lies far below the safety threshold defined for enological applications (Ivanova-Petropulos, Wiltsche, Stafilov, Stefova, Motter, & Lankmayr, 2013). Regardless of the ambiguity in the definition of a common risk threshold for the Pb content, the hypothesis of occasional contaminations occurring along the supply chain would require a detailed investigation in a representative number of samples to be confirmed, and the implementation of purification processes.

4. Conclusions

In this work, the composition of commercially available food-grade tannins was explored to achieve two objectives: (i) identify markers for the authentication of commercial products according to the declared botanical origin, and (ii) monitor the presence of potentially toxic contaminants. Objective (i) was achieved through the combined use of MALDI-TOF MS and UV-Vis spectrophotometric methods which showed variable degree of purity and variable percentage of tannins; in particular, the highest content in polyphenols was reached by the SKP extract (93.3%), followed by LOE > GTP > SEP. The quantitative analysis of polyphenols accounted for the antiradical activity of the extracts, which reflects the nutritional properties of additives. According to the chemical composition of the polyphenolic fraction the main discriminant fingerprints between the plant extracts evaluated as authentication tools were found: the composition in flavonoid and their degree of galloylation for SEP, SKP and GTP samples, and the specific glycosylation patterns and castalagin/vescalagin derivatives occurring in the LOE sample. The GTP sample showed a high variety of molecular fragments attributable to flavonols, which have a high antioxidant power and may legitimize the use

of this extract as a food supplement. Recognition patterns were detected for the GTP sample (628 Da protonated fragment) and for the LOE extract (sodium adduct of roburin dimer at 1873 Da); the two molecular fingerprints were suggested as authenticity markers for the tea leaves and *Quercus* wood extracts. In objective (ii), the ICP-MS elemental profile confirmed the levels of potentially toxic contaminants; Pb levels were found to exceed the level suggested by the WHO regulations for drinking water, despite higher concentrations are allowed in other food applications. The present work has highlighted the need for correct production practices along the supply chain, to minimize the occurrence of external contamination sources. To our knowledge, the elemental composition of a Limousin oak food-grade tannin was reported for the first time.

The present work constitute a preliminary study, and will be implemented increasing the number and variety of samples to build a database of commercial products currently available in the market, thus contributing to their proper exploitation in the food industry.

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Figures captions

Figure 1_ The MALDI-TOF MS spectrum of sample SEP (linear positive mode, ion gate: 400 Da) recorded in the range 650–2500 Da: procyanidins series (repeat unit: catechins) for the grape seed extract.

Figure 2_ The MALDI-TOF MS spectrum of sample SKP (linear positive mode, ion gate: 400 Da) recorded in the range 650–2500 Da: procyanidins series (repeat unit: catechins) for the grape skin extract.

Figure 3_ The MALDI-TOF MS spectrum of sample GTP (linear positive mode, ion gate: 200 Da) recorded in the range 650–2500 Da: glycosylated chain series.

Figure 4_ The MALDI-TOF MS spectrum of sample LOE (linear negative mode, ion gate: off) recorded in the range 400–650 Da showed basic units of ellagitannins and glycosylation patterns (see **Table 5**).

Tables

Table 1_ Phytochemical composition and antioxidant capacity of selected commercial tannins. Results from replicates (n = 3) are reported as mean values ± standard deviation. Legend: grape seed (SEP) and skin (SKP) proanthocyanidins, green tea procyanidins (GTP), Limousin oak ellagitannin (LOE).

	Tannin samples			
	SEP	**SKP**	**GTP**	**LOE**
Total polyphenols (mM CE[a])	1.62 ± 0.12	2.83 ± 0.01	2.11 ± 0.01	2.34 ± 0.03
Tannins (mM CE)	1.49 ± 0.02	1.23 ± 0.01	1.04 ± 0.02	1.42 ± 0.0
*Tannins/total polyphenols (%)	93.8	42.9	47.6	60.9
DPPH (mM TE[b])	0.26 ± 0.0	0.24 ± 0.01	0.42 ± 0.01	0.39 ± 0.01
[#]Non-phenolic compounds* (%)	46.7	6.7	30.0	23.3

[a]Expressed as (+)-catechin equivalent. [b]Expressed as Trolox equivalent.

*Calculated as % weight: tannins/total polyphenols.

[#] Calculated as % weight: 1 g/L dry powder – estimated total polyphenols.

Table 2_ Calculated and experimental MALDI-TOF peaks related to the galloylated procyanidins series in the SEP sample.

DP*	**no. of galloyl units (+ 152 Da)**	**Calculated ($M+Na^+$)**	**Observed($M+Na^+$)** *Linear positive mode*
Trimer	0	890	891
	1	1041	_
	2	1194	1194
	3	1344	1344
Tetramer	0	1178	1178
	1	1330	1328
	2	1482	1482
	3	1634	1633
Pentamer	0	1466	_
	1	1618	_
	2	1770	_
	3	1922	1922
	4	2074	2080
Hexamer	0	1754	_
	1	1906	_
	2	2058	_
	3	2210	_
	4	2363	_
Heptamer	0	2042	2037
	1	2194	2197
	2	2347	2348
	3	2495	2497
	4	2939	_
Octamer	0	2331	_
	1	2483	2480
	2	2635	_
	3	2787	_
	4	2939	_

*DP: degree of polymerisation. (–) Not present.

Table 3_ Calculated and experimental MALDI-TOF peaks for procyanidins detected in the SKP sample.

Calculated (M, Da)	**Observed (M, Da)**	**Attribution**
	Linear positive mode	
542	544	Fisetinidin dimer
572	572	Catechin-gallocatechin with loss of water
578	581	A- /B- type procyanidins
591	592	Catechin-gallocatechin dimer
604	600	B-type procyanidin, ($+Na^+$)
633	634	Gallocatechin-gallocatechin dimer ($+Na^+$)
715	713	Catechin gallate + catechin dimer, with loss in -OH
823	828	Gallocatechin + catechin + flavanol fragment, trimer
847	847	Gallocatechin + catechin + flavanol fragment,trimer ($+Na^+$)
1111	1114	Gallocatechin + 2*catechin + flavanol fragment, tetramer
1129	1131	2*gallocatechin + catechin + flavanol fragment, tetramer
Oligomers with increase in catechin unit (+288 Da)		
1419	1415	Pentamer
1707	1702	Hexamer
1995	1990	Heptamer
2300	2298	Octamer (+ gallocatechin*)

*Octamer, gallocatechin as a possible terminal unit.

Table 4_ Calculated and experimental MALDI-TOF peaks for flavonol and flavonol glycosides detected in the GTP sample.

Calculated (M, Da)	**Observed (M, Da)**	**Attribution**
	Linear positive mode	
309	309	Kaempferol (+ Na^+)
318	318	Myricetin
325	325	Quercetin (+ Na^+)
448	444	Kaempferol-3-glycoside (astragalin)
464	464	Quercetin-3-glycoside (isoquercetin)
510	510	Myricetin-3-glycoside (+ Na^+)
610	609	Quercetin-3-rutinoside (rutin)
617	617	Kaempferol-3-rutinoside (+ Na^+)
847	847	Quercetin-3-rutinoside (+ Na^+)

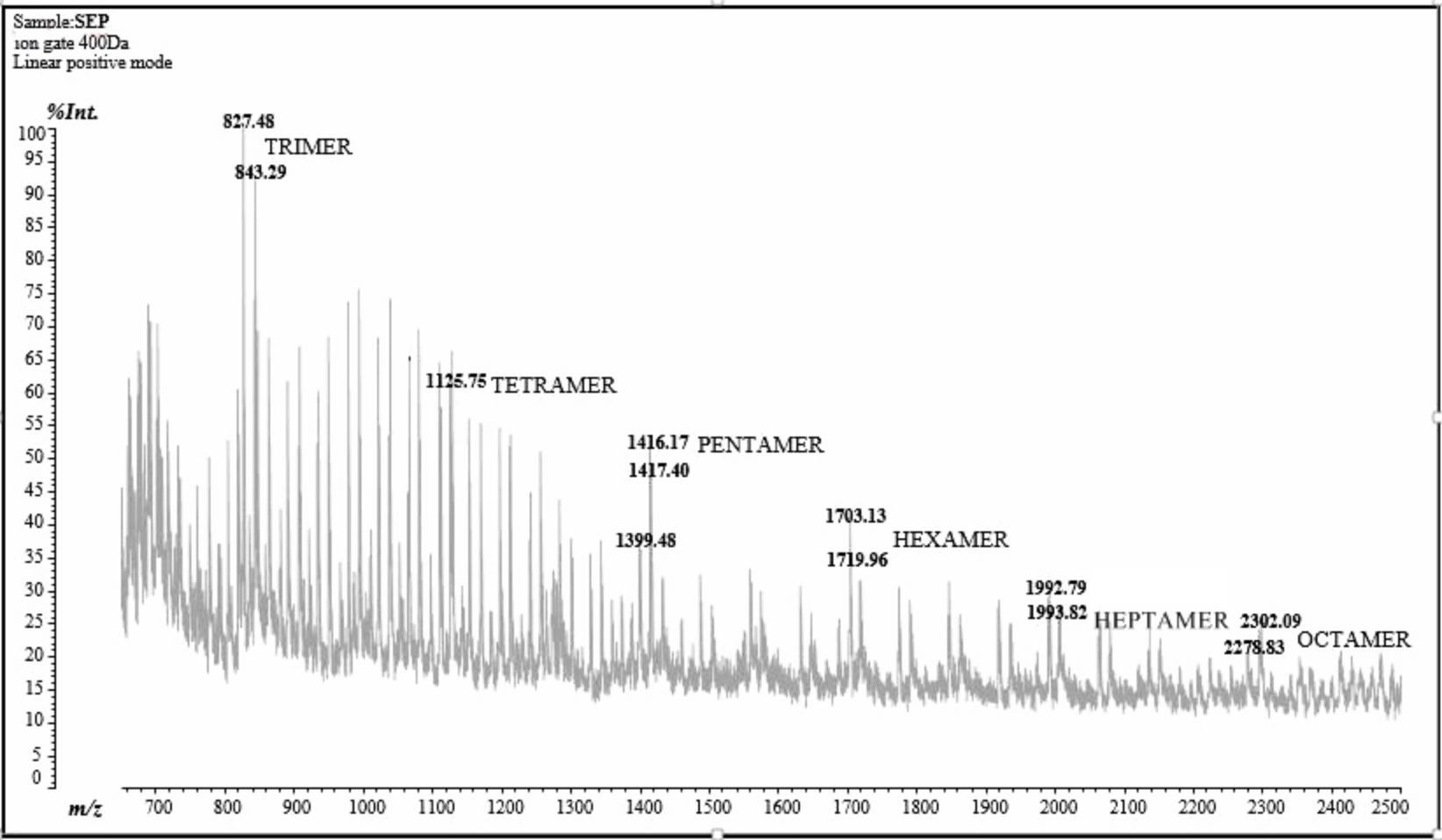
Table 5_ MALDI-TOF fragmentation patterns for ellagitannins and polygalloylglucoside compounds detected in the LOE sample.

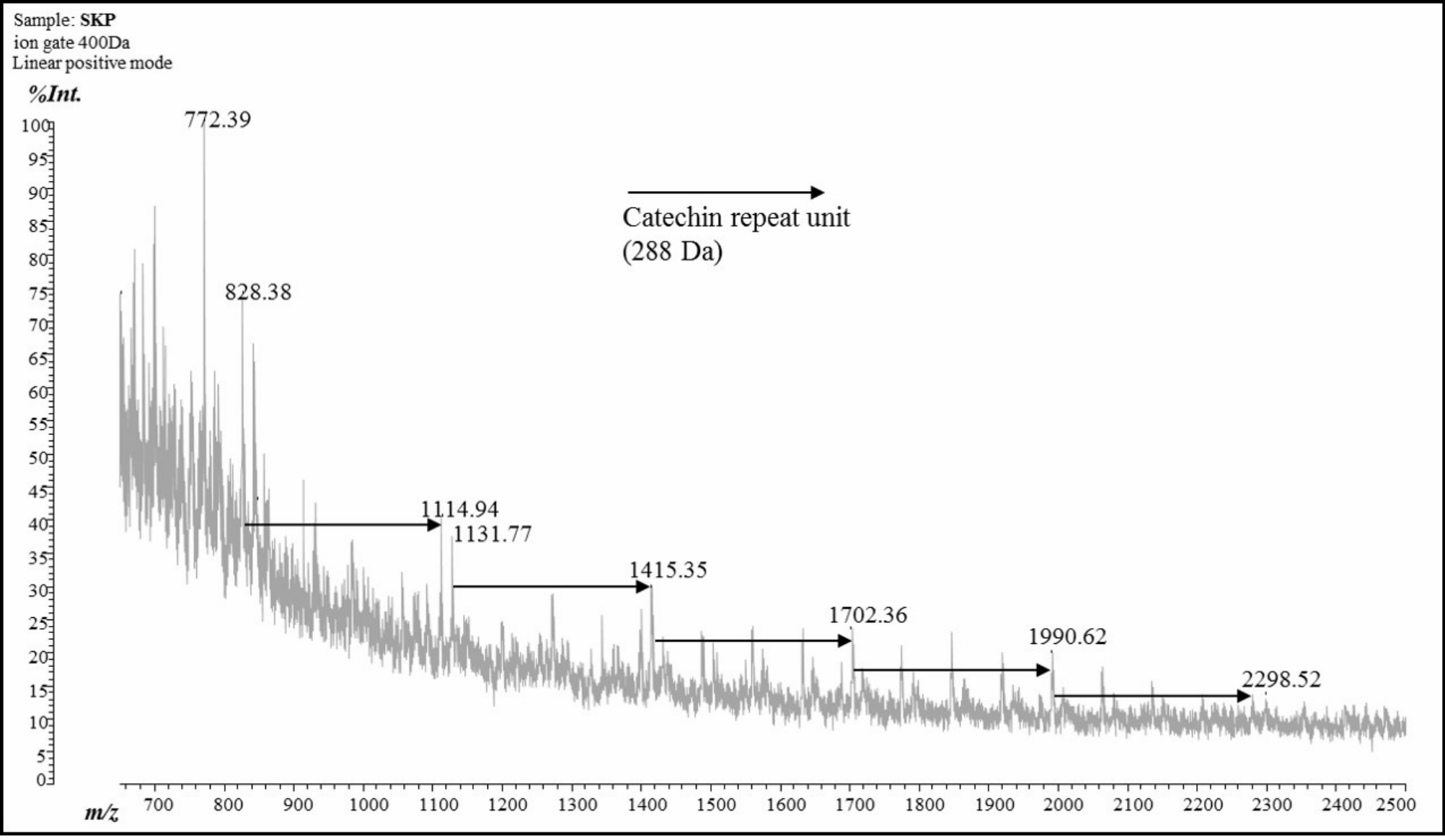
Calculated (M, Da)	Observed (M, Da)	Attribution
	Linear negative mode	
364	362	Gallic acid dimer + sugar fragment
390	394	Galloyl moiety + sugar + sugar fragment
424	424	Gallic acid dimer + sugar fragment
463	463	Ellagic acid hexoside
493	494	Valoneic acid dilactone (+ Na^+)
507; 506	506	Digalloyl glucose (+ Na^+); Valoneic acid
567	566	Gallic acid trimer + sugar fragment (+ Na^+)_loss in two -OH
634	633–634	Galloyl HHDO glucose
1035	1038	Esamer: 2 gallic acids and 4 hexosyl polysaccharide + glucose residue
1463	1463	Castalagin or pentagalloylglucose linked to a nonahydroxythriphenoic acid (+Na^+)

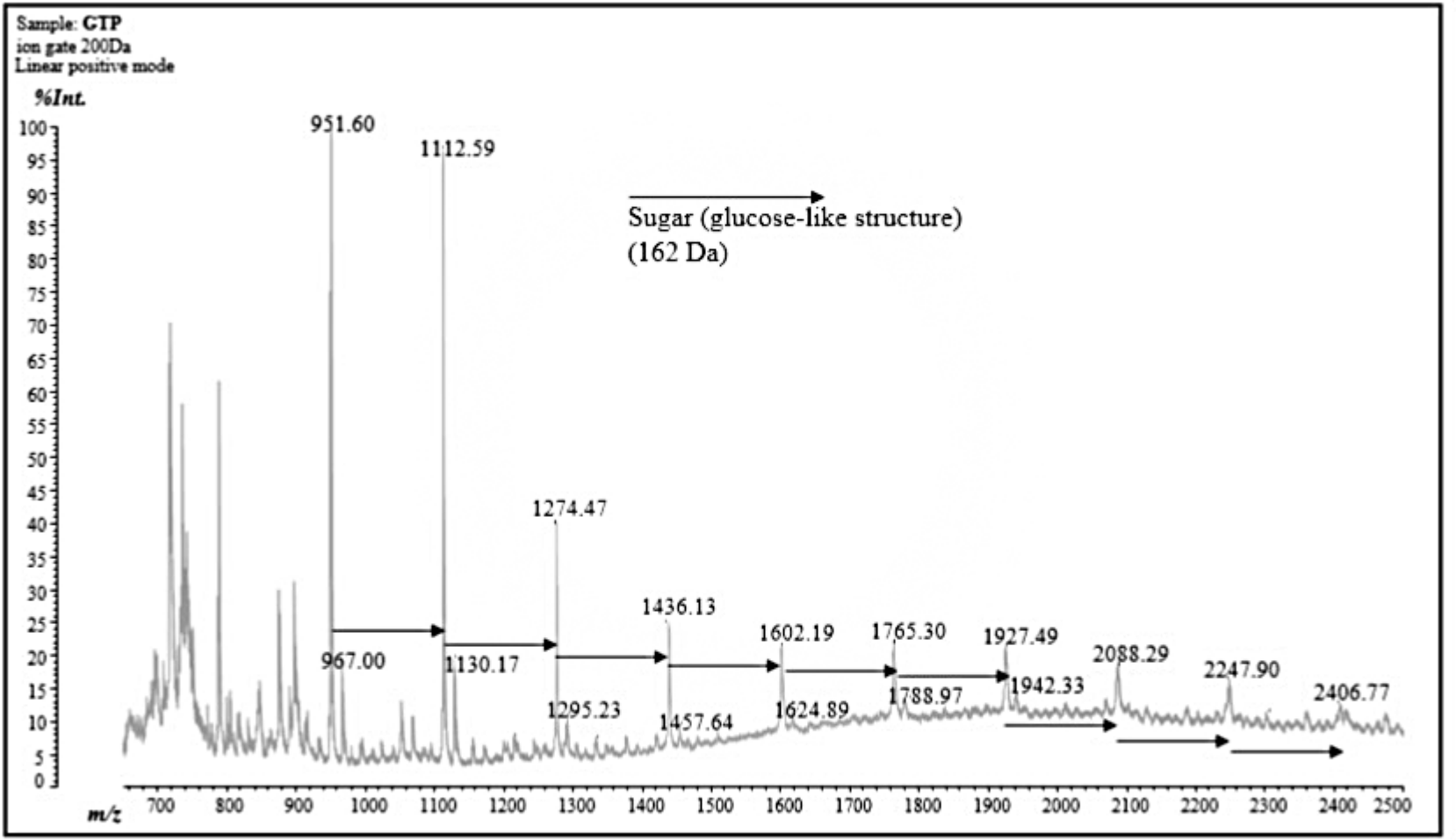
Table 6_ Elemental composition of selected commercial tannins used as food additives; results are provided as mean values (n=3).

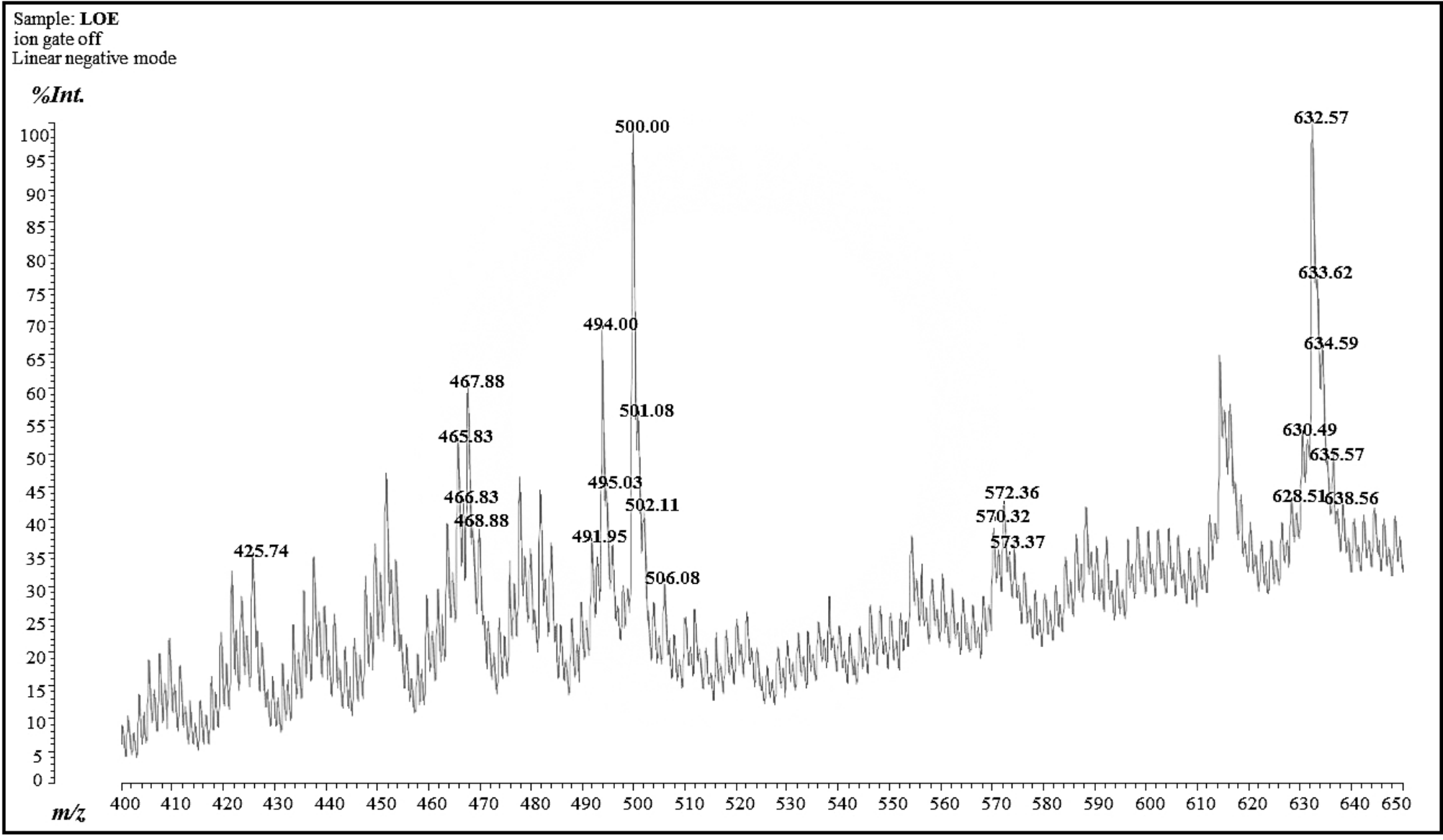
	Tannin samples (ppm)				(ppm)	(%)	**Guideline values (WHO, 2004; ppm)**
Element	**SEP**	**SKP**	**GTP**	**LOE**	**LOD**	**RSD***	
^{7}Li	0.001	0.001	0.002	0.002	8.53E–06	2.44	na
^{24}Mg [He]	0.080	0.090	0.500	0.200	1.34E–04	1.64	na
^{27}Al	nd	nd	nd	nd	1.01E–03	2.48	0.200
^{39}K [He]	1.400	1.200	0.800	0.600	2.31E–04	1.39	na
^{44}Ca [He]	0.200	0.220	0.400	0.400	7.81E–04	2.28	na
^{52}Cr [He]	0.009	0.011	0.008	0.008	9.09E–06	1.89	0.050
^{55}Mn [He]	0.001	0.002	0.010	0.004	5.81E–06	1.96	0.400
^{56}Fe	0.050	0.050	0.060	0.030	9.77E–05	2.28	1.000-3.000
^{59}Co	0.0001	0.0001	0.0001	0.0001	3.30E–07	2.03	na
^{60}Ni	nd	nd	nd	nd	7.95E–05	1.94	0.070
^{63}Cu	0.020	0.020	0.040	0.020	1.35E–05	2.06	2.000
^{66}Zn	nd	nd	0.990	0.250	5.51E–04	1.95	3.000
^{75}As	0.002	0.002	0.002	0.002	1.30E–05	2.52	0.010
^{88}Sr	0.003	0.004	0.020	0.020	2.22E–05	1.96	na
^{137}Ba	0.010	0.010	0.020	0.020	7.02E–04	2.17	0.700
^{207}Pb	0.031	0.033	0.033	0.033	5.74E–05	1.61	0.010

RSD*: Average values for each element. nd: not detected or below the quantitation limit; na: not available.


Sample:SEP
ion gate 400Da
Linear positive mode
%Int.
827.48
TRIMER
843.29
1125.75 TETRAMER
1416.17 PENTAMER
1417.40
1399.48
1703.13
HEXAMER
1719.96
1992.79
1993.82
HEPTAMER
2302.09
2278.83
OCTAMER
m/z


Sample: SKP
ion gate 400Da
Linear positive mode
%Int.
772.39
828.38
1114.94
1131.77
1415.35
1702.36
1990.62
2298.52
Catechin repeat unit
(288 Da)
m/z


Sample: GTP
ion gate 200Da
Linear positive mode
%Int.
951.60
1112.59
Sugar (glucose-like structure)
(162 Da)
1274.47
1436.13
1602.19
1765.30
1927.49
2088.29
2247.90
2406.77
967.00
1130.17
1295.23
1457.64
1624.89
1788.97
1942.33
m/z


Sample: LOE
ion gate off
Linear negative mode
%Int.
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
m/z
400 410 420 430 440 450 460 470 480 490 500 510 520 530 540 550 560 570 580 590 600 610 620 630 640 650
500.00
632.57
633.62
494.00
634.59
467.88
501.08
465.83
630.49
635.57
495.03
466.83
572.36
628.51
638.56
502.11
468.88
570.32
491.95
573.37
425.74
506.08