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Screening and identification of major phytochemical compounds in seeds, sprouts and leaves of Brassica oleracea L. var. acephala

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Keywords:	Brassica oleracea, seed, sprouts, phytochemical composition, antioxidant activity, molecular fingerprintings

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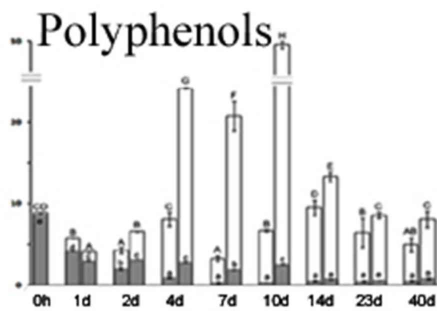

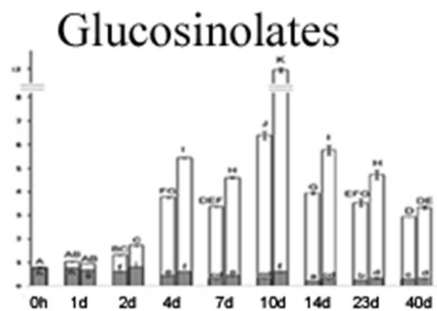

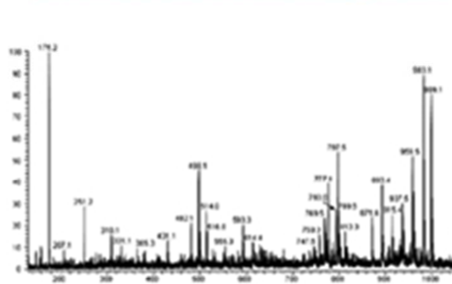

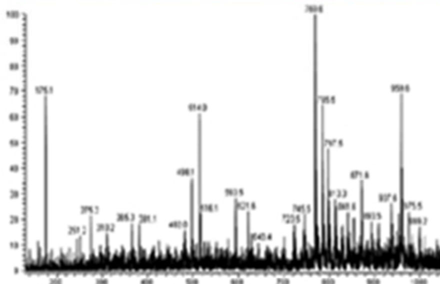

ESI(+) mass spectra in seeds and leaves

44x43mm (300 x 300 DPI)

Screening and identification of major phytochemical compounds in seeds, sprouts and leaves of *Brassica oleracea* L. var. *acephala*

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ABSTRACT

Brassica oleracea var. *acephala* (kale) was investigated for total polyphenols, flavonoids, glucosinolates and antioxidant activity in seeds, seedlings and edible leaves. Molecular fingerprints, identification and characterization of phytochemical compounds were obtained by mass spectrometry (MS), high resolution MS and tandem MS with electrospray ionization in positive and negative ion modes. The maximum amount of phytochemical compounds, spectrophotometrically determined, was observed in 10 days sprouts. Total antioxidant activity was maximum in 2, 4 days seedlings. The main phenolic compounds identified by MS were the flavonoid caffeoylglucose, the hydroxycinnamic acid sinapine and the glucosinolates glucoerucin and glucobrassicin. The antioxidants ω3 and ω6 were observed in leaves. The identification of the stages with maximum phytochemical compounds can encourage the consumption of kale sprouts and young leaves in the diet. Our research can support food industries to maximize the health-promoting properties of the sprouts or pharmaceutical industries for the production of food supplements.

KEYWORDS

Brassica oleracea, seed, sprouts, phytochemical composition, antioxidant activity, molecular fingerprintings

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36 **1. Introduction**

37 Vegetables are central components of the human diet, as they contain fibers, vitamins, minerals,
38 antioxidants and can be consumed in large quantities for their low calorie intake. Many studies
39 demonstrated that dietary habits based on regular intake of vegetables can prevent diabetes,
40 cardiovascular and neurological diseases and decrease the risk of many forms of cancer (Shetty et
41 al. 2013; Rautiainen et al. 2015). In particular, vegetables belonging to the *Brassicaceae* family
42 (cabbage, kale, cauliflower, broccoli, Brussels sprouts) are recognized for their health benefits.
43 Their active components are mainly represented by polyphenols, flavonoids, vitamins E and C and
44 glucosinolates, all with antioxidant and anti-inflammatory properties, which contribute to
45 chemopreventive activity in colon, stomach and lung cancer (Soengas et al. 2011).

46 Phenolic compounds are secondary metabolites produced during plant development, with well
47 known beneficial effects against chronic diseases (Boivin et al. 2009). Among polyphenols, mainly
48 flavonoids can exert protective action on diseases through antioxidant activity as scavengers against
49 peroxyl and hydroxyl radicals. They act reducing low-density lipoproteins oxidation (Rankin et al.
50 1993; Cao et al. 1997), modulating cell signaling, interacting with cell receptors, enzymes and
51 transcription factors (Williams et al. 2004).

52 Glucosinolates are the most important class of secondary metabolites peculiar to *Brassicaceae*
53 with protective action against pathogens (antibiotic and insecticidal activities) and deterrent action
54 against herbivores attacks (Brown & Morra 1995; Smith 2000). In the environment they may act as
55 allelochemicals, affecting plant communities and controlling plant growth in close proximity (Fahey
56 et al. 2001). In animals, glucosinolates and their hydrolysis products isothiocyanates, are well
57 known protectors against the development of many forms of cancer (Lynn et al. 2006), capable also
58 to block chemical carcinogenesis (Pan et al. 2017). This suggests that great intake of *Brassicaceae*
59 may have a chemoprotective effect and lower the risk of several types of diseases (Wu et al. 2009).

60 Previous works analyzed the nutritional value of many *Brassicaceae* plants mainly considering
61 the phytochemical composition of seed oil (Cacciola et al. 2016), leaves or other edible portions in
62 fresh tissues, or after cooking or storing (Korus & Lisiewska 2011; Bongoni et al. 2014).

63 The phytochemical composition of *Brassicaceae* varies considerably as a consequence of
64 developmental stages and species considered (Scialabba et al. 2010); previous studies reported that
65 edible sprouts of several *Brassicaceae* including broccoli and cauliflower had 10–100 times higher
66 levels of the glucosinolate glucoraphanin than the corresponding mature plants, even though in
67 many crucifers the content of glucosinolates and phenolic compounds was greatest in seeds when
68 analyzed on fresh weight basis (Baenas et al. 2012).

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Brassica oleracea var. *acephala* (kale) is a green leafed cabbage without head largely cultivated in Europe and in North America. Kale has curly leaves that are consumed cooked, for the thickness of stem and leaves. The young and tender leaves of kale, easily consumable in fresh salad, are recently commercialized at the stage of 40 days, registered with the name of Kavoli®. However, few information was reported up to now on the variation of phytochemicals in kale from seeds to sprouts and leaves. The aim of this research was therefore to assess, firstly by spectrophotometric methods, the relative level of total polyphenols, total flavonoids, glucosinolates and antioxidant activity in different portions of seedlings (cotyledons and embryonic axes) and of plantlets (cotyledons and leaves) in order to determine the presence and variation of secondary metabolites and antioxidant capacity during different stages of kale development up to 40 days of cultivation.

In metabolomics studies, mass spectrometry is widely used being a very effective methodology for identifying, characterizing and quantifying unknowns. High sensitivity, high selectivity and high specificity are some of its main features (Lei et al. 2011; Araujo et al. 2005). In the present study, molecular fingerprints of the samples analyzed spectrophotometrically, were determined by electrospray ionization with direct infusion of ethanolic extracts with no purification, coupled to high resolution (HRMS) and tandem mass spectrometry (MS^n). This gave a clear and immediate picture of complexity and variation in molecular composition of metabolome in different samples**.**

2. Results and discussion

2.1 Dry weight determination

The DW was determined for each kale sample from seeds to leaves (Fig. 1A). A rapid and progressive water absorption during seed imbibition and germination was observed, so that DW decreased significantly from 0.97 g DW/ g FW in seeds to 0.12 g DW/ g FW in seedlings at 4 days of germination. Afterwards, DW ranged between 0.05 and 0.1 g DW/ g FW in plants from 7 to 40 days with no significant differences. The first step of seed germination is represented by water uptake (imbibition) with subsequent activation of enzymes that stimulate metabolic activity, radicle emergence (Bewley et al. 2013) and seedling growth. Expressing the content of molecules on a fresh or dry weight basis could alter the interpretation of the nutritional significance and health benefit of the material (Lim 2012). For this reason, water content determination in plant tissues may allow the actual quantification of the molecules of interest in respect to fresh and dry weight.

2.2 Total polyphenols content

The highest total polyphenols content was detected in seeds (8.6 mg GAE/g FW) (Fig. 1B). After 1 day of imbibition, total polyphenols content was higher in embryonic axes than in cotyledons (4.1

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mg GAE/g FW and 3 mg GAE/g FW respectively). From 2 days of imbibition up to seedlings of 10 days, cotyledons always showed a total polyphenols content significantly higher than embryonic axes. From 14 up to 40 days no significative differences in the content of total polyphenols were observed between cotyledons and embryonic axes or leaves, the amount always ranging around 1-2 mg GAE/g FW. Plants grown in sprouters at 40 days have values of total polyphenols comparable to plants grown in the field (Kavolì). When expressed on DW basis, total polyphenols increased significantly in cotyledons from 4 to 10 days, up to 49.2 mg GAE/g DW, twice than in 4 days cotyledons, 8 times than in tissues of 1-2 days and 10 times than in leaves of 40 days old plants. This concentration corresponds to 253 mg GAE/100g FW, almost 10 times higher than that reported in several other *Brassicaceae* (Singh et al. 2007) where the range was 12.6-34.4 mg /100 g FW. However, among *Brassicaceae* the maximum amount of phenolic compounds was reported in broccoli in function of different varieties: 44.5 - 82.9 mg/100 g FW (Singh et al. 2007), 101.6 mg/100 g FW (Chu et al. 2002), 337 mg/100 g FW (Podsędek 2007).

2.3 Flavonoids content

The highest flavonoids content was detected in seeds (6.7 mg QE/g FW) (Fig. 1C). The content was high in early stage of seedlings (5.5 mg QE/g FW in embryonic axis and 2.7 mg QE/g FW in cotyledons at 1 day of imbibition). At 2 days of imbibition, the content was higher in cotyledons than in embryonic axis (3.1 mg QE/g FW and 2.5 mg QE/g FW respectively) and kept more or less constant in further stages of seedling development. On DW basis, the flavonoids content was significantly higher in cotyledons than in embryo axis from 4 days of sprouting, with the highest value at 10 days (38.4 mg QE/g DW), that is 5.5 times higher than in seeds (7 mg QE/g DW) and 4.9 times higher than in leaves from 40 days old plants (7.9 mg QE/g DW). A consistent amount of flavonoids was detected also in embryonic axes at most of the stages investigated. Although colorimetric methods here adopted can sometimes lead to an underestimation of some classes of flavonoids (Chang et al. 2002), the amount detected in kale exceeded that of other vegetables and *Brassicaceae* already reported (Korus & Lisiewska 2011). The increase in flavonoids during sprouting of kale, partially recalling the polyphenols presence, can be explained taking into account that the former compounds are a major group of the polyphenols family with antioxidant activity.

2.4 Glucosinolates content

On FW basis, major glucosinolates content was in seeds and cotyledons at 2 days of imbibition (Fig. 1D); the content decreased in all samples from 14 days onwards. On DW basis the values of glucosinolates increased from 4 days of sprouting, reaching a maximum in cotyledons at 10 days of

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11.9 mg GRFSE/g DW, which was 15 times higher than in seeds (0.8 mg GRFSE/g DW) and 3.6 higher than in leaves of 40 days old plants (3.3 mg GRFSE/g DW). With the exception of 10 days, glucosinolates content in cotyledons from 4 days of sprouting up to 40 days ranged from 3 to 6 mg GRFSE/g DW, whereas in embryo axis values ranged around 3-4 mg GRFSE/g DW. Previous works demonstrated that the glucosinolates concentration depends on plant growth conditions (climatic factors, soil fertility, water stress) (Rangkadilok et al. 2004) and on plant developmental stage, with different amount in leaves, root, stem, seed (Del Carmen Martínez-Ballesta et al. 2013). The decrease reported in our results over germination time were similarly reported by Baenas et al. (2012) for several *Brassicaceae* sprouts, where the decrease was explained by the role of glucosinolates as plant defense and nutrient reserve compounds, mainly in seeds.

The present results evidence that kale is a richer source of phytochemical compounds than other Brassica vegetables (Chu et al. 2002). The recommended dietary allowance (RDA) for glucosinolates is 12-16 mg/day (Verhoeven et al. 1997); the presence of a large number of structurally different polyphenols impairs to set an RDA for these compounds; although a general consensus of opinion suggests an optimum intake between 650 mg/day (Zamora-Ros et al. 2013) and 1 mg/day (Landete 2013). In kale, total polyphenols, total flavonoids, glucosinolates and total antioxidant activity were highest in seedlings at 10 days of cultivation, but also 40 days old plants, already commercialized as Kavolì, much more cost-effective in respect to seedlings, contained good levels of the considered phytochemical compounds.

2.5 DPPH Radical Scavenging Activity*

The antioxidant activity of fresh tissues, expressed as percent inhibition of the DPPH* radical, was high in seeds (Fig. 1E), and increased both in embryonic axes and in cotyledons during germination up to 2 days. Hereafter, the antioxidant capacity dropped up to 10 days, being however significantly higher in cotyledons than in embryonic axes; from 14 to 40 days it kept constant in leaves and cotyledons, being about 6 times lower than in cotyledons from 2 days old seedlings. The increase of antioxidant activity during early stages of kale germination and the subsequent decrease with germination was also reported in different cultivars of broccoli and radish seeds (Martinez-Villaluenga et al. 2010). This raise may be related to the increase in antioxidant compounds such as vitamins and polyphenols (Fernandez-Orozco et al. 2006).

2.6 Metabolomic fingerprinting by mass spectrometry

To qualitatively determine the metabolomic fingerprints and their variations in different parts of kale, a mass spectrometry approach, consisting in the use of electrospray ionization in positive (Fig.

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170 S1) and a negative ion mode (Fig. S4), high resolution and tandem mass spectrometry (Fig. S2, Fig. S3 and Fig. S4), has been developed and used in this study. Putative attributions of compounds based on high resolution mass spectrometry and tandem mass spectrometry are reported in Table 1 and Table 2. The main naturally occurring phenolic compounds identified were the flavonoid caffeoylglucose, the hydroxycinnamic acid sinapine and the glucosinolates glucoerucin and glucobrassicin. Moreover the presence of the antioxidant ω3 and ω6 should be emphasized.

176 The mass spectra evidence a significant change in metabolic profiles as a function of plantlet development.

178

179 3. Conclusions

180 Despite the wide use of sprouts in raw products, few information was available to date on kale antioxidant content and potentially beneficial effects. The present results demonstrate that kale have a consistent amount of compounds with well known positive properties on health and high antioxidant activity, so that their use in the diet should be encouraged to increase the content of antioxidant in the diet. Although among *Brassicaceae* kale is a minor crop in many parts of the world, the present results evidence that it is a good source of nutritive compounds. The analysis of this work was conducted on seedlings and of plantlets expressed on fresh and dry weight basis, with the aim to dissect the individual contribution of cotyledons, embryonic axes and leaves. The identification of the plant tissues and the developmental stages with the major quantity of phytochemicals compounds could be a support of information for food industries to maximize the health-promoting properties of the sprouts or for pharmaceutical industries for the production of food supplements.

192

193 Captions to the Figures

194

195 **Fig. 1.** (A) g dry weight/g of fresh material at different stages of growth in kale from seeds (0h) to plantlets (40 days). (B) Total Polyphenols content expressed as mg gallic acid equivalents (GAE mg/g). (C) Flavonoids content expressed as mg quercetin equivalents (QE mg/g). (D) Glucosinolates content expressed as glucorafasatine equivalents (GRFSE mg/g). (E) DPPH assay: antioxidant activity expressed as percentage of antiradical activity (ARA %).

200 All determinations were done at the same stages of seed imbibition, plant sprouting and development in fresh (FW ■) or dry (DW □) tissues. B-E. Different letters denote significant differences ($p < 0.05$) according to Tukey's test between data at different times of growth. Upper

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203 and lowercase letters represent dry and fresh tissues, respectively. Bars are the mean ± SE (n = 3) of
204 different tissue extracts. Embryonic axes (ea); cotyledons (cot); leaves (le).

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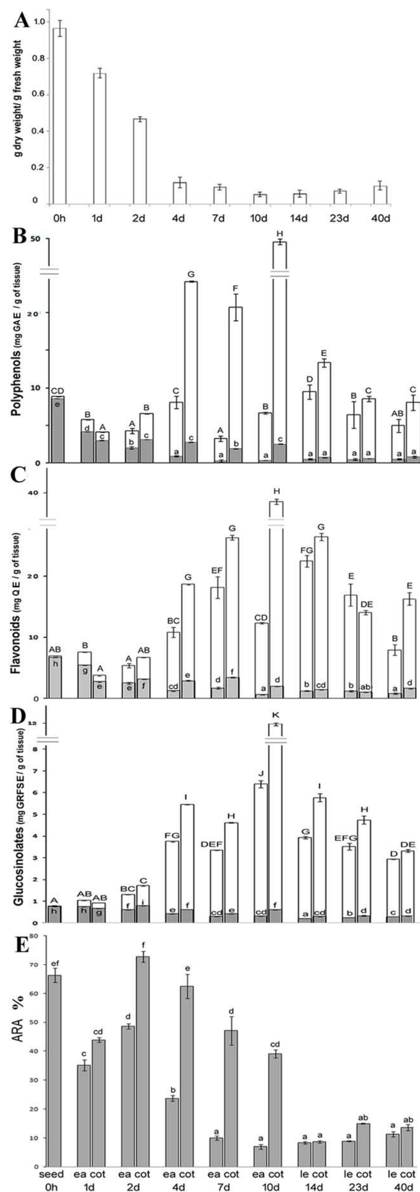

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All determinations were done at the same stages of seed imbibition, plant sprouting and development in fresh (FW ▪) or dry (DW ▫) tissues. B-E. Different letters denote significant differences ($p < 0.05$) according to Tukey's test between data at different times of growth. Upper and lowercase letters represent dry and fresh tissues, respectively. Bars are the mean ± SE (n = 3) of different tissue extracts. Embryonic axes (ea); cotyledons (cot); leaves (le).

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Table 1. ESI(+) data with putative identification of most significant ions

m/z	Attribution	MW	Compound
251, 207, 147	Fragment ions (see text)		Sinapine
175	*in seeds* two isobaric ions: fragment ion of sinapine (90%) and protonated arginine (100%)		Sinapine and arginine
	in cotyledons and leaves: fragment ion		Sinapine
310	$[M+H]^+$	310	Sinapine
365, 381	$[M+Na]^+$, $[M+K]^+$	342	Disaccharide
482, 498, 514	$[MNa+Na]^+$, $[MNa+K]^+$, $[MK+K]^+$	437	Glucoraphanin
527, 543	$[M+Na]^+$, $[M+K]^+$	504	Trisaccharide
593, 615, 631	$[M+H]^+$, $[M+Na]^+$, $[M+K]^+$	592	Pheophorbide A/fragment ions of Pheophytin
689, 705	$[M+Na]^+$, $[M+K]^+$	666	Tetrasaccharide
769, 785	$[M+Na]^+$, $[M+K]^+$	746	MGDG 34:6
777, 793	$[M+Na]^+$, $[M+K]^+$	732	Disinapoyldisaccharide
797, 813	$[M+Na]^+$, $[M+K]^+$	774	DGDG36:6
871, 893, 909	$[M+H]^+$, $[M+Na]^+$, $[M+K]^+$	870	PheophytinA
937, 959, 975	$[M+H]^+$, $[M+Na]^+$ $[M+K]^+$	936	DGDG 36:6
983, 999	$[M+Na]^+$, $[M+K]^+$	960	Trisinapoyldisaccharide

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Table 2. ESI (-) data (all detected species are due to [M-H]$^{-}$).

m/z	MW	Compound
223	224	Sinapinic acid
241	242	Pentadecanoic acid (C15:0)
253	254	Palmitoleic acid (C16:1)
255	256	Palmitic acid (C16:0)
277	278	Linolenic acid (C18:4)
279	280	Linoleic acid (C18:3)
281	282	Oleic acid (C18:2)
283	284	Stearic acid (C18:0)
337	338	Erucic acid (C22:1)
341	342	Disaccharide
385	386	Sinapoyl-monosaccharide (possibly glucose)
420	421	Glucoerucin
436	437	Glucoraphanin
447	448	Glucobrassicin
477	478	Neoglucobrassicin
671	672	PEth32:2
673	674	PEth32:1
675	676	PEth32:0
693	694	PEth34:5
695	696	PEth34:4
697	698	PEth34:3
699	700	PEth34:2
701	702	PEth34:0
719	720	PEth36:6
721	722	PEth36:5
723	724	PEth36:4
725	726	PEth36:2
727	728	PEth36:1
737	738	PEth 37:4
739	740	PEth 37:3
751	752	PEth38:4
753	754	Disinapoyldisaccharide
743	744	PG 34:4
745	746	PG 34:3
747	748	PG 34:2
831	832	PI C34:4
833	834	PI C34:2
835	836	PI C34:0

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Screening and identification of major phytochemical compounds in seeds, sprouts and leaves of *Brassica oleracea* L. var. *acephala*

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ABSTRACT

Brassica oleracea var. *acephala* (kale) was investigated for total polyphenols, flavonoids, glucosinolates and antioxidant activity in seeds, seedlings and edible leaves. Molecular fingerprints, identification and characterization of phytochemical compounds were obtained by mass spectrometry (MS), high resolution MS and tandem MS with electrospray ionization in positive and negative ion modes. The maximum amount of phytochemical compounds, spectrophotometrically determined, was observed in 10 days sprouts. Total antioxidant activity was maximum in 2, 4 days seedlings. The main phenolic compounds identified by MS were the flavonoid caffeoylglucose, the hydroxycinnamic acid sinapine and the glucosinolates glucoerucin and glucobrassicin. The antioxidants ω3 and ω6 were observed in leaves. The identification of the stages with maximum phytochemical compounds can encourage the consumption of kale sprouts and young leaves in the diet. Our research can support food industries to maximize the health-promoting properties of the sprouts or pharmaceutical industries for the production of food supplements.

KEYWORDS

Brassica oleracea, seed, sprouts, phytochemical composition, antioxidant activity, molecular fingerprintings

Experimental

Plant materials

Brassica oleracea L. var *acephala* (kale) seeds were kindly provided by “Consorzio Freschissimi, Campagna Lupia, Venezia, Italy”.

Seeds germination and seedlings growth conditions

Kale seeds were rinsed in distilled water, then sown in Petri dishes with three sheets of Whatman filter papers imbibed with a diluted Hoagland solution (1:1 in distilled water) (Hoagland & Arnon 1950). Seeds were germinated at 24 °C in the dark for 4 days, then exposed to day light until the 10th day for the analysis of the earlier stages of germination and growth. Twenty seeds per dish, for a total of 100 seeds, were sown for every analysis. Another aliquot of seeds (100 seeds) were placed into a sprouter (30 x 70 mm) filled with vermiculite, kept for 4 days in the dark at 24 °C for germination, then transferred to day light for a total of 40 days to obtain seedlings and plantlets at

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later developmental stage. The sprouts were wet every other day with a diluted Hoagland solution (1:1 in water). Antioxidant assays were carried out on dehulled seeds (time 0) and on seedlings, sprouts and plantlets, i.e. at 1, 2, 4, 7, 10 days on cotyledons and embryonic axes and at 14, 23 and 40 days on cotyledons and young leaves separately. All the determinations were performed using a PERKIN ELMER, INC. UV/Vis spectrophotometer.

Dry weight determination

For every determination 1 g of fresh plant material (FW) was weighed, dried in an oven at 102°C overnight to constant weight, weighed again to obtain dry weight (DW) and expressed as g DW/ g FW.

Extractions

One g of each tissue of kale was homogenized with an Ultra-turrax homogenizer in 10 ml of 80% ethanol on ice, then centrifuged for 30 min at 1130 x *g* at 4°C to recover the supernatant. The pellet was subjected to a second extraction and the supernatant was added to the first extraction. Extracts were immediately processed or stored in a refrigerator (-20°C) and analyzed within 1 month.

Determination of total phenolic content

The total polyphenols content of the different kale extracts was determined by a colorimetric assay based on the procedure of Singleton & Rossi (1965). In particular, 100 µl of extract were added to 3 ml of diluted Folin-Ciocalteau (Sigma) (5-fold diluted with distilled water). After 6' of incubation, 2 ml of 20% sodium carbonate solution was progressively added dropwise and stirred. After 1 hour of incubation at room temperature (RT), absorbance was determined at 760 nm against a blank. The polyphenols content was estimated through the calibration curve of gallic acid and expressed as mg of gallic acid equivalents (GAE /g FW or DW) of extract.

Determination of total flavonoid content

The total flavonoids content of kale extracts was estimated using a colorimetric method (Heimler et al. 2006). In particular, 200 µl of extract was mixed with 60 µl of 5% $NaNO_2$ solution, 800 µl of distilled water and incubated at RT for 5 min. Then, 60 µl of $AlCl_3$ 10% in water was added and incubated for 6 min. Next, 400 µl of 1M NaOH and 480 µl of distilled water were added. The mixture was allowed to stand for 5 min at RT before the absorption was measured at 510 nm against a blank. The flavonoids content was estimated through the calibration curve of quercetin and expressed as mg quercetin equivalents (QE /g FW or DW) of extract.

Determination of glucosinolates

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The glucosinolates content of kale extracts was estimated at 365 nm using a colorimetric method described by Fahey et al. (1997). 500 µl of extracts were first evaporated to dryness and dissolved in 200 µl of distilled water. Glucosinolates were quantitatively converted to isothiocyanates by enzymatic treatment with 20 µl myrosinase 28U/ml for 1 h at 37°C. Then 100 µl of 80 mM 1,2-benzenedithiol were added to 900 µl of methanol and 780 µl of 0.1 M potassium phosphate buffer and incubated at 64°C for 1 h. This step allowed the cyclocondensation of isothiocyanates with 1,2-benzenedithiol to generate 1,3-benzedithiol-2-thione (ε of 23,000 $M^{-1}cm^{-1}$ at 365nm). The glucosinolates content was estimated through the calibration curve of glucorafasatine (GRFS) and expressed as GRFS/g FW or DW of extract.

DPPH assay*

Antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as described by Boudjou et al. (2013). A solution of 60 µM DPPH (1950 µl) in methanol was mixed with 50 µl of the different kale extracts, then vortexed and kept at 25 °C in the dark for 60 min. Absorbance at 517 nm was measured using methanol as a blank. As control, 50 µl of ethanol were used instead of extract. Antiradical activity (ARA) was expressed as percentage inhibition of the DPPH***** radical, determined by the following equation:

ARA = 100 x (1 – (absorbance of sample/absorbance of control)).

Mass spectrometry analysis

Kale samples were analyzed by electrospray ionization in positive and negative ion mode on a LCQ DECA ion trap (ThermoFinnigan, Bremen, D) at the following conditions: spray voltage (+/-) 4.5 kV; capillary temperature 200°C; sheath gas (nitrogen) flow rate, ca. 0.75 L/min. MS^n product ion experiments were carried out inside the ion trap by isolating the precursor ion and then by applying a supplementary potential for collision induced dissociations; collision gas: He; collision energy: 24-30% arbitrary units (a.u.). The *m/z* window for precursor ion isolation was 1 or 2 u. Extracts diluted with methanol were introduced via flow injection in the ESI source at a flow rate of 5 µL/min.

The Orbitrap Q Exactive Plus (Thermo Fisher, Bremen, D) with an electrospray source, operating in positive and negative ion mode, in flow injection, was used for high resolution (30000 and 140000 FWHM@*m/z* 200) mass spectra and higher-energy collisional dissociation (HCD) MS/MS spectra by using nitrogen as collision gas; collision energy: 18-30% arbitrary units.

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ChemSpider (Royal Society of Chemistry) and Phenol-Explorer 3.5 (French National Institute for Agricultural Research) have been used as auxiliary tools for the identification of the compounds.

Statistical analysis

All the experiments were repeated three times and data reported as mean ± SE. For phytochemicals determination three extracts from a pool of at least 20 individual samples (seeds, embryos, cotyledons and leaves) were analyzed. Analysis of variance (ANOVA) and a *post hoc* Tukey's multiple range test were used to identify statistically significant differences between treatments using the Statistica package (StatSoft) 6.0 version.

Mass spectrometry analysis

Positive ion mode

The ESI(+) spectra of seeds, 10 and 23 days cotyledons and 40 days leaves extracts are compared in Fig. S1. Most of the ions are due to cationized molecules with sodium ($[M+Na]^+$) or potassium ($[M+K]^+$) ions and only in a few cases to protonated molecules ($[M+H]^+$). The mass spectra evidence a significant change in metabolic profiles as a function of plantlet development. In particular, in seeds (Fig. S1A) low molecular weight metabolites constitute the most abundant peaks in the ESI(+) mass spectrum. Abundant ions are at *m/z* 310. Based on HRMS and MS^n experiments they have been attributed to sinapine (accurate mass *m/z* 310.1645, error -1.1 ppm). Sinapine is an alkaloid found in some seeds, particularly oil seeds of plants belonging to the family of *Brassicaceae* (Nićiforović & Abramovič 2014), considered as an acetylcholinesterase inhibitor with potential therapeutic applications in different diseases (He et al. 2008; Ferreres et al. 2009).

The structure of sinapine is further confirmed by the MS^2 spectrum obtained by selecting ions at *m/z* 310, which shows a prominent product ion at *m/z* 251 due to loss of $N(CH_3)_3$. On turn, it decomposes in MS^3 experiments to ions at *m/z* 207 ($-C_2H_4O$), 175 ($207-CH_3OH$) and 147, due to a further loss of CO, in full agreement with the structure of sinapine. All these ions are present and with high abundances in the ESI(+) mass spectrum of seeds extract (Fig. S1A). So it is supposable that sinapine undergoes a spontaneous fragmentation inside the ESI ion source yielding fragment ions at *m/z* 147, 175, 207 and 251 in the ESI(+) mass spectrum.

The use of HRMS allows to separate and differentiate isobaric ions. Indeed it is interesting to zoom in the ions at *m/z* 175 in the mass spectra of seeds, cotyledons and leaves. It appears that two isobaric ions, at *m/z* 175.0385 and 175.1185, respectively, are observed in seeds, while only one peak is present in the mass spectrum of cotyledons and leaves at any of the considered time (Fig. S2). The former are attributed to $C_{10}H_7O_3$ (error -2.7 ppm), due to spontaneous fragmentation of

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sinapine in the ion source, while the latter (*m/z* 175.1185, $C_6H_{15}O_2N_4$, error -2.6 ppm), is attributed to protonated arginine, in agreement with its MS/MS spectrum.

Carbohydrates are also detectable in the ESI (+) mass spectra. In fact the couples of ions at *m/z* 365/381, 527/543 and 689/705 are attributable to $[M+Na]^+$, $[M+K]^+$ adducts of di-, tri- and tetrasaccharides, respectively. They are quite intense in the mass spectrum of seed extract, while only ions due disaccharides are detectable in the leaves extract. Ions at *m/z* 365 are detectable also in the mass spectrum of cotyledons at day 10 (Fig. S1B).

At higher *m/z* values, a triplet of ions is at *m/z* 482, 498 and 514 attributable to sodium and potassium adducts, and in particular to $[MNa+Na]^+$, $[MNa+K]^+$, $[MK+K]^+$, respectively, of glucoraphanin which has been confirmed by HRMS and MS/MS experiments. These ions are present in all the ESI (+) mass spectra, but *m/z* 482 is lacking in 23 days cotyledons.

Polyphenols are also detectable as sodium or potassium adducts. In fact in the mass spectrum of seeds and cotyledons of 10 days, ions at *m/z* 777 and 793 are well detectable. HRMS has allowed to assign the molecular formulae as $[C_{34}H_{42}O_{19}Na]^+$ (error -1.4 ppm) and $[C_{34}H_{42}O_{19}K]^+$ (error -1.5 ppm) respectively, which have been tentatively assigned to the sodium and potassium adducts of a disinapoyldisaccharide, such as disinapoylgentiobiose, disinapoylsucrose, etc. Trisinapoyl-disaccharides are also detectable at *m/z* 983 and 999 as sodium and potassium adducts, respectively, with high intensity in the mass spectrum of the cotyledons extract (Fig. S1B, C). The MS^2 spectrum of ions at *m/z* 983 shows elimination of 224 Da ($C_{11}H_{12}O_5$) giving rise to *m/z* 759 which, on turn, decomposes by losses of water, $C_{11}H_{12}O_5$ and $C_{17}H_{20}O_9$, as shown by its MS^3 spectrum (data not shown). By considering literature data, these ions should be ascribed to 1,2,2, trisinapoylgentiobiose but other trisinapoyldisaccharides should be equally present.

Analysing cotyledons and leaves the high *m/z* range becomes more populated of ions (Fig. S1B, C, D). In the ESI(+) mass spectrum of cotyledons extracts intense ions are at *m/z* 871 ($[C_{55}H_{75}N_4O_5]^+$, error -0.8 ppm), 893 and 909 assigned to protonated, sodium and potassium adducts of pheophytin A, respectively. These ions have a less intensity in the mass spectrum of leaves while they are undetectable in that produced by the seed extract.

The MS^2 and MS^3 spectra (Fig. S3) of precursors at *m/z* 871 and 593 evidence the structure of pheophytin A, which is formed from the chlorophyll molecule, with substitution of two Mg^{++} with two H^+ and serves as the primary electron acceptor in photosystem II (Klimov 2003). It is noteworthy that the main decomposition of ions at *m/z* 871 yields ions at *m/z* 593 (Fig. S3A), attributed to protonated pheophorbide A produced by the elimination of phytol. This reaction is analogous to that catalyzed by chlorophyllase which hydrolyzes the ester bond of chlorophyll to yield chlorophyllide and phytol. Ions at *m/z* 593 further decompose to product ions at *m/z* 575, 565,

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561 and 533, due to losses of water, CO, CH_3OH and CH_3COOH, respectively (Fig. S3B). The mass spectrum of the cotyledons extract shows ions at *m/z* 593 highly abundant at 23 days, also detectable in leaves extract, together with ions at *m/z* 615 and 631 due to sodium and potassium adducts, respectively (Fig. S1C, D). The presence of pheophorbide A might be ascribed to its spontaneous fragmentation inside the ESI ion source and/or to some degradation reactions of pheophytin A. In fact, it has been reported that the decomposition of chlorophyll a and chlorophyll b into phaeophytin and pheophorbide occurs during leaves senescence (Schelbert et al. 2009). In the present research this pathway may occur during cotyledons aging as confirmed by the higher amount of pheophytin A and pheophorbide A in 23 days than 10 days cotyledons.

Glycolipids, producing intense ions in cotyledons and highly intense in leaves extracts are also detectable in their ESI(+) spectra (Fig. S1B, C). In fact for ions at 769 (*m/z* 769.4855) the chemical formula $[C_{43}H_{70}O_{10}Na]^+$ (error -0.8 ppm) has been attributed and its tandem mass spectrum shows eliminations of $C_{16}H_{26}O_2$ and $C_{18}H_{30}O_2$ suggesting a monogalactosyldiacylglycerol (MGDG 34:6) esterified with hexadecatrienoic acid (C16:3) (ω3) and octadecatrienoic acid (linolenic acid, C18:3) (ω6). Ions at *m/z* 785 are due to its potassium adduct, present in cotyledons of 23 days and in leaves (Fig. S1C, D, respectively). Another glycolipid has been found at *m/z* 797 $[M+Na]^+$ and *m/z* 813 $[M+K]^+$, attributed to a MGDG 36:6, in which the two ester chains are two molecules of linolenic acid. A digalactosyldiacylglycerol (DGDG 36:6) esterified with two molecules of linolenic acid was also detected at *m/z* 937 $[M+H]^+$, 959 $[M+Na]^+$ and 975 $[M+K]^+$, with high intensity in the leaves extract (Fig. S1D).

Galactolipids (MGDGs and DGDGs) are a class of compounds widely found in the plant kingdom, including edible plants, and are an important part of the cell membranes. Numerous studies have shown that galactolipids derived from plants, cyanobacteria and green algae exhibit various biological properties *in vitro* and/or *in vivo*, including anti-tumor activity (Hou et al. 2007), anti-inflammatory activity (Larsen et al. 2003) and antiviral activity (Reshef et al. 1997).

Negative ion mode

The ESI(–) mass spectra of seeds and cotyledons extracts are depicted in Fig. S4. Operating in electrospray in negative ion mode, polar substances with acidic groups, such as glucosinolates and polyphenols, can be detected. While for the seeds extract peaks are well evident till over *m/z* 1000, in the case of cotyledons and leaves extracts there are too many peaks of almost the same intensity, with high background, making difficult the distinction between signal and noise.

The ESI (-) spectra of both seeds and cotyledons extracts are dominated by the peak at *m/z* 436, assigned by MS^2 and MS^3 spectra to deprotonated glucoraphanin. In fact, deprotonated molecule

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3 $[M-H]^-$ of intact glucosinolates produces characteristic fragment ions at *m/z* 259 and 195 which have been assigned to the sulfated glucose and the thioglucose anions, respectively (Maldini et al. 2016). In seeds another glucosinolate was identified at *m/z* 420 as glucoerucin. Other two glucosinolates are glucobrassicin (*m/z* 447) and neoglucobrassicin (*m/z* 477).

Ions in the low *m/z* range have been assigned to anions of fatty acids, and in particular due to pentadecanoic (C15:0), palmitoleic (C16:1), palmitic (C16:0), linolenic (C18:4), linoleic (C18:3), oleic (C18:2), stearic (C18:0) and erucic (C22:1) acids (Table 2).

Sinapinic acid (*m/z* 223) and its derivatives, *i.e.* sinapoyl-monosaccharide (*m/z* 385, possibly glucose) and disinapoyldisaccharide (*m/z* 753, possibly saccharose) are also detected.

Ions at *m/z* 341 might be due to caffeoyl glucose and/or to disaccharide anions. High resolution measurements showed the presence of only one peak at *m/z* 341.1088 attributed to a disaccharide anion ($C_{12}H_{21}O_{11}$, error = -0.45 ppm).

Noteworthy is the presence of ions in the 671-751 *m/z* range (Fig. S5A). Their *m/z* values and accurate masses are in agreement with phosphatidic acids with different fatty acids. As an example, ions at *m/z* 697.4819, 699.4973, 701.5127, 721.4816 and 723.4974 are attributed to $C_{39}H_{70}O_8P^-$ (error 0.81 ppm), $C_{39}H_{73}O_8P^-$ (error 0.46 ppm), $C_{39}H_{74}O_8P^-$ (error -0.03 ppm), $C_{41}H_{70}O_8P^-$ (error 0.39 ppm), $C_{39}H_{72}O_8P^-$ (error 0.57 ppm) (Table 2). On the other hand, the MS/MS spectra are not in agreement with the structures of phosphatidic acids having a $ROPO_3^-$ moiety. Let us consider, as an example, the ESI(–) HCD (20% a.u.) MS/MS spectra of ions at *m/z* 699 in the cotyledons 10 days extract (Fig. S5B). The product ions are at *m/z* 461, 437, 419, attributed to losses of $O{=}C{=}CHC_{14}H_{29}$, $O{=}C{=}CHC_{16}H_{29}$, $HOOCC_{17}H_{31}$ (C18:2) from $[M-H]^-$, respectively, while those at *m/z* 279, 255 are due to the $C_{18}H_{31}O_2P^-$ (C18:2) and $C_{16}H_{31}O_2P^-$ (C16:0) species, respectively. The sum of the carbon atoms of the fatty acids observed in the fragment ions gives a total of 34 carbons, while they must be 36. A deeper insight into the high resolution HCD MS/MS spectrum shows further product ions at *m/z* 124.9998 and 181.0263 attributed to $C_2H_6O_4P^-$ and $C_5H_{10}O_5P^-$, suggesting the presence of an ethyl group on the phosphate moiety. It follows that, even if they molecular weights and chemical compositions of these compounds are in full agreement with phosphatidic acids, indeed they are phosphatidylethanols (PEth). PEths is a group of aberrant phospholipids formed in cell membranes in the presence of ethanol by the catalytic action of the enzyme phospholipase D on phosphatidylcholine (Gnann et al. 2009; Nalesso et al. 2011). So it is reasonable that the use of ethanol in the extraction procedure caused the formation of PEth derivatives.

Another series of glycerolphospholipids has been identified as phosphatidylinositols at *m/z* 831, 833, 835 attributed to PI C34:4, PI C34:2 PI C34:0, respectively (Fig. S4).

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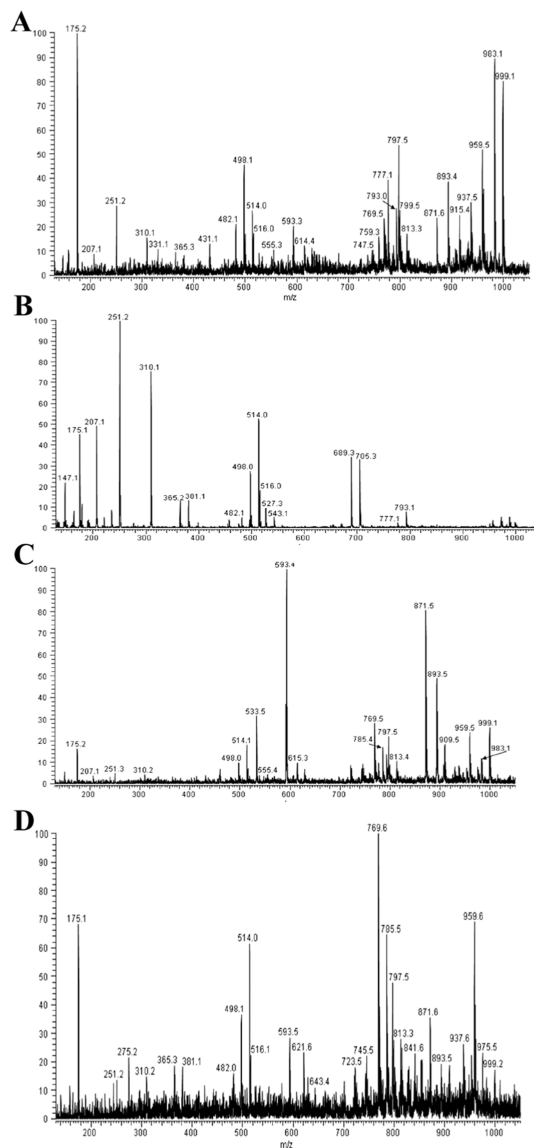

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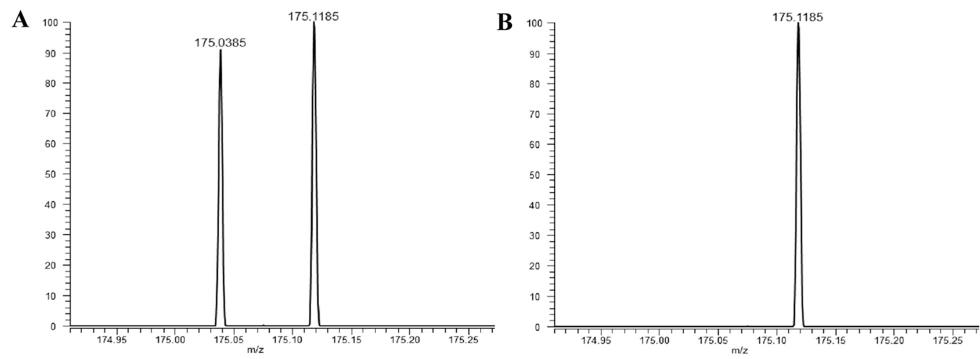

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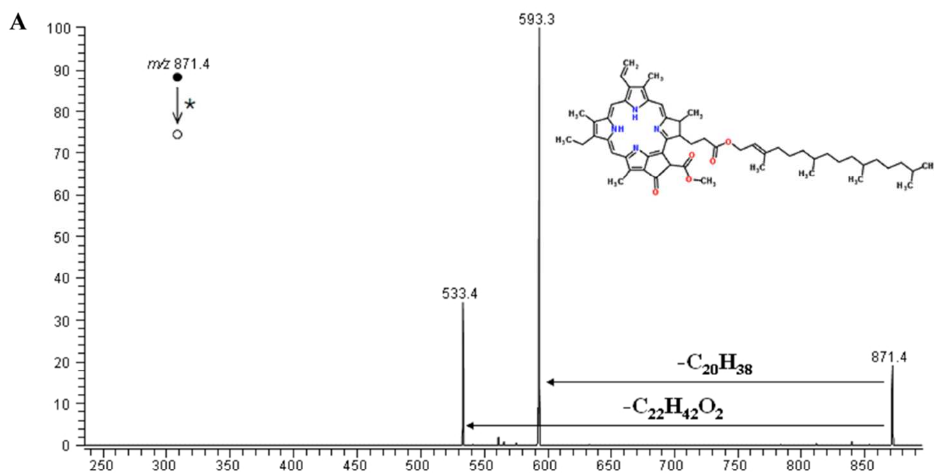

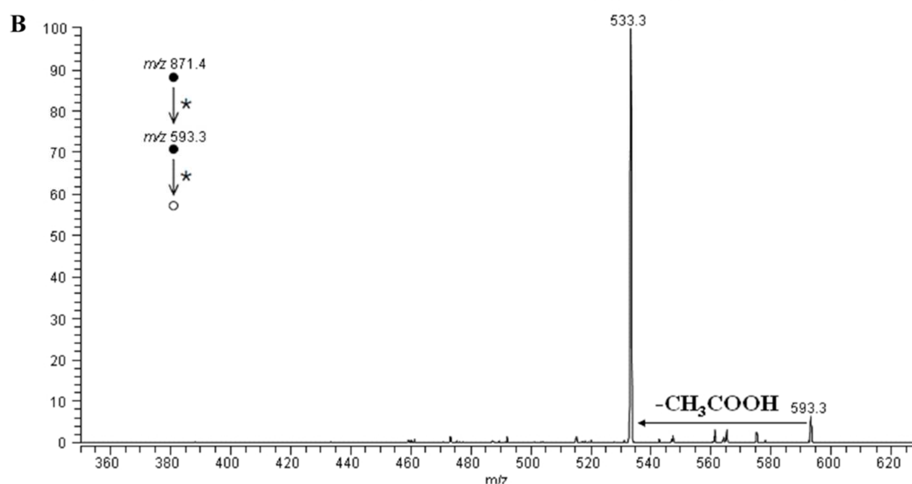

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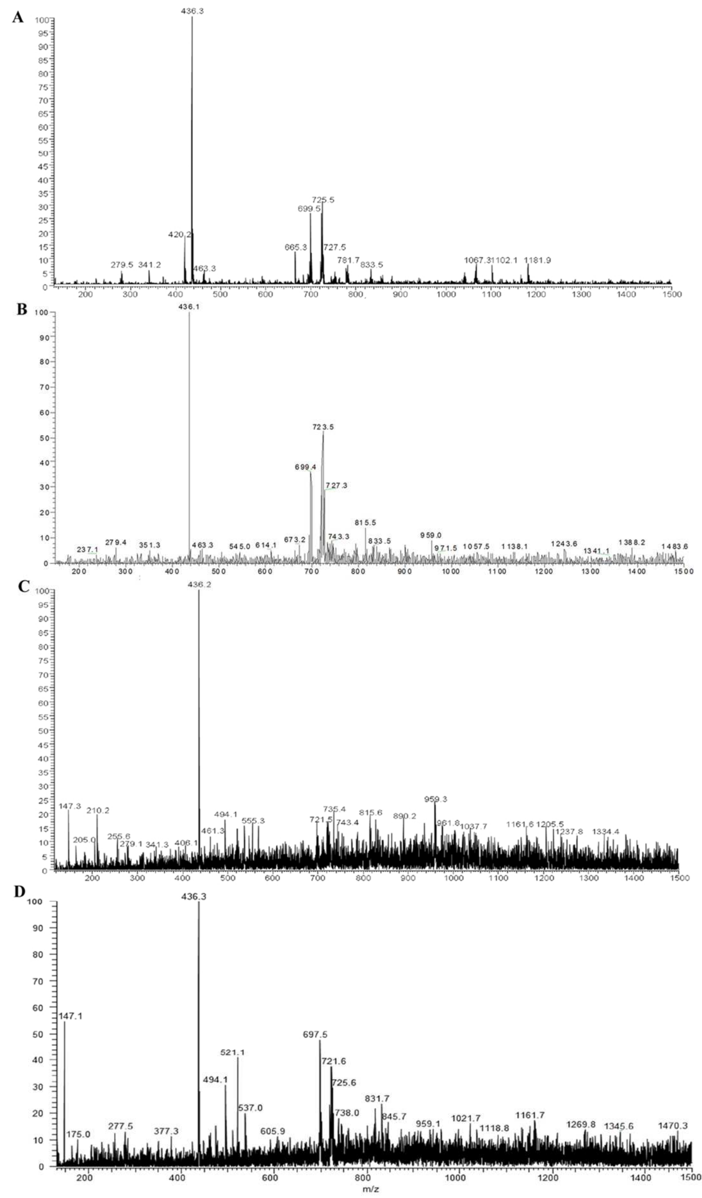

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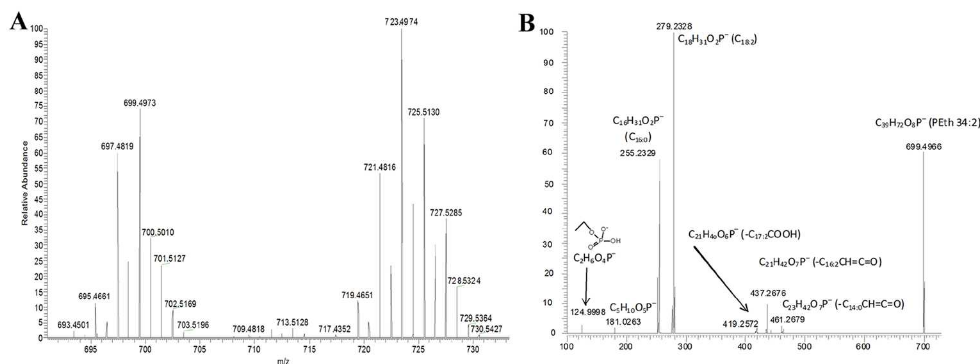

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