FOODINTEGRITY
Ensuring the Integrity of the European food chain

613688: Collaborative Project

Seventh Framework Programme
KBBE.2013.2.4-01: Assuring quality and authenticity in the food chain

Deliverable: 14.1

Title: Report on development of chemical and metabolomic markers of product integrity and stability along processing.

Author(s): Baroni, María Verónica; Erban, Alexander; Kopka, Joachim; Wunderlin, Daniel

Beneficiary(s): Food Integrity Consortia

Date of preparation: September 21th 2017.

Period covered: November-2016; August-2017

Status: version 1

Dissemination level		
PU	Public	X
PP	Restricted to other participants	
RE	Restricted to a group specified by the consortium	
CO	Confidential, only members of the consortium	

The project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement No. 613688.

Deliverable 14.1 Report on development of chemical and metabolomic markers of product integrity and stability along processing.

TABLE OF CONTENTS **Page Nr.**

Deliverable 14.1 Report on development of chemical and metabolomic markers of product integrity and stability along processing.

1 Description of Deliverable

This deliverable reports procedures used to evaluate chemical and metabolomic markers of the integrity of nutritive seeds (chia, line and sesame). These markers were evaluated by different methods at two different laboratories (MPIMP and CONICET-ICYTAC). Results demonstrate that it is possible using a set of chemical compounds from both volatile and non-volatile fractions as markers of authenticity. Current results enable a clear identification of each seed by a set of chemical markers, which can be used to prevent fraud at any food industry using these seeds within their products, and by consumers acquiring these seeds for their own nutrition. Preliminary results also demonstrate that chemical/ metabolic markers can be used to identify the presence of these nutritive seeds in bakery products, which needs to be fully validated and presented in future deliverables (14.2 - 14.3).

2 Achievement of the Deliverable

2.1 Chemical fractionation and profiling of seeds for the discovery of potential chemical markers by GC-MS (MPIMP).

2.1.1. Seed selection (EU market).

MPIMP collected a test sample set of chia (Salvia hispanica L.), flax (linseed; Linum usitatissimum L.), sesame (Sesamum indicum L.) seed batches that were sold for human consumption autumn 2016 in local grocery stores (Berlin, Germany) or were purchased via internet sources. The available data of the 28 seed batches are listed with in supplemental table S1 (Food Integrity Samples_Seed_Collection_MPIMP.xlxs). The seed test sample set comprised 8 independently marketed seed batches of mostly brown chia seeds, and 4 independently marketed batches of predominantly off-white chia seeds, golden or brown linseeds and white or black sesame seeds (Figure 1). All seed batches, except one white sesame sample contained the seed coats. No seed batch was defatted or otherwise visibly processed. All seed batches were photo-documented and all information given on the packaging archived.

Figure 1. Pictures of test samples for seed species used for the search of specific chemical and metabolomic marker. Seed batches were collected in Berlin (Germany), where they are marketed for human consumption. The colour code is used in subsequent analyses. The number of independently marketed replicates is given in square brackets.

2.1.2 Fractionation scheme to evaluate chemical markers in seeds:

The seeds were subjected to several GC-MS metabolite profiling methods that were established by partner MPIMP. In detail, samples were directly subjected to organic volatile carbon (VOC) emission analysis by (1) headspace solid phase micro-extraction gas chromatography – mass spectrometry (Agudelo-Romero et al., 2013), or to liquid extraction followed either by (2) GC-MS profiling of a water methanol fraction of predominantly polar metabolites (Erban et al., 2007; Dethloff et al., 2015) or (3) by GC-MS profiling of metabolic components from a predominantly lipophilic fraction after chemical hydrolysis that was developed for terpenoid and fatty acid profiling (Fuentes et al. 2016). The remaining solid fraction was (4) hydrolysed and profiled using a method that was established for analysis of carbohydrate and non-carbohydrate cell wall components (Gal et al., 2016). The separation scheme covered all major compound classes that can be monitored by GC-based profiling methods (Figure 2).

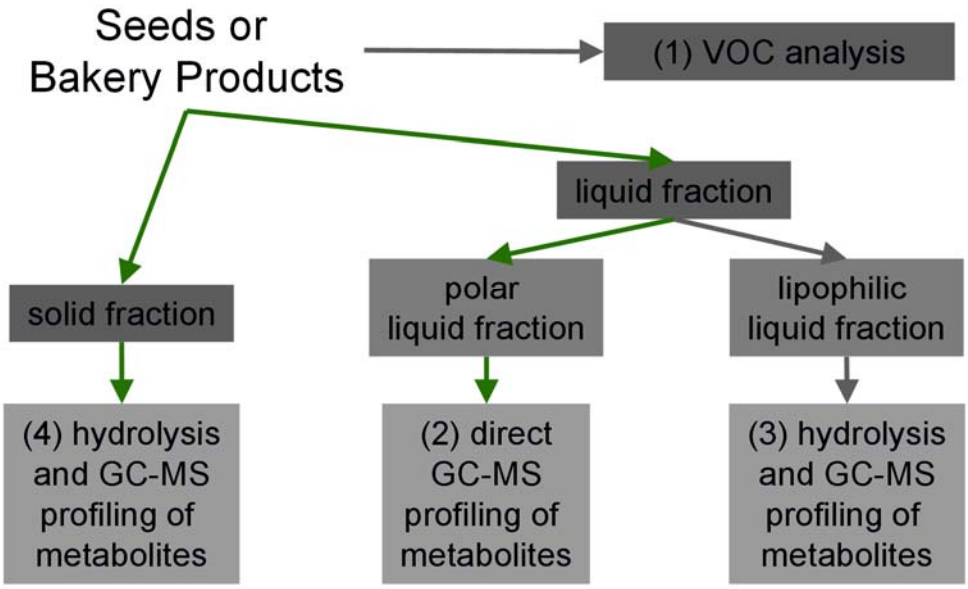


Figure 2. Chemical fractionation and profiling scheme of seed or seed containing material for the discovery of potential chemical markers. VOC analysis is intended for seed batch analysis prior to processing only. Chemical profiling methods that were adapted are listed within the text and among the references.

The analysis of volatile organic compounds (VOC; Figure 2-(1)) was intended to evaluate seed batches prior to seed processing, considering that the production of food includes heating processes, which can modify the volatile profile or induce loss of seed specific chemicals. Compositional analysis of terpenoids and fatty acids of the liquid extractable lipophilic fraction was attempted (Figure 2-(3)), but did not yield useful marker information. Further attempts were abandoned considering that terpenoids were of low abundance compared to the fatty acids. Additionally, in many cases food processing involves the use of lipids form other sources than the original seed ingredients. Thus, fatty acids do not qualify as good markers of the presence of seeds in a complex food product. Therefore, we focussed on the profiling of the predominantly polar fraction (Figure 2-(2)) or, alternatively, on the hydrolysate of the solid residue after liquid extraction (Figure 2-(4)). The profiling of these two fractions, in addition to VOCs profile from seed material, provided candidates of chemical markers of studied seeds, which will be used for future SOP validation (deliverable 14.2). The presence of such markers was deduced from the data mining described below.

2.1.3 Evaluation of chemical markers in seeds by GC-MS and chemometrics:

Data mining of candidates extracted and analysed by GC-MS as described above was performed by principal component analyses (PCA) of non-targeted metabolite profiles (Figure 3). Markers were independent of the seed colour. The sum of total variance

captured by PC1 and PC2 of each PCA and the number of technical replicate analyses of each of the 28 seed batches are given in the legend of Figure 3.

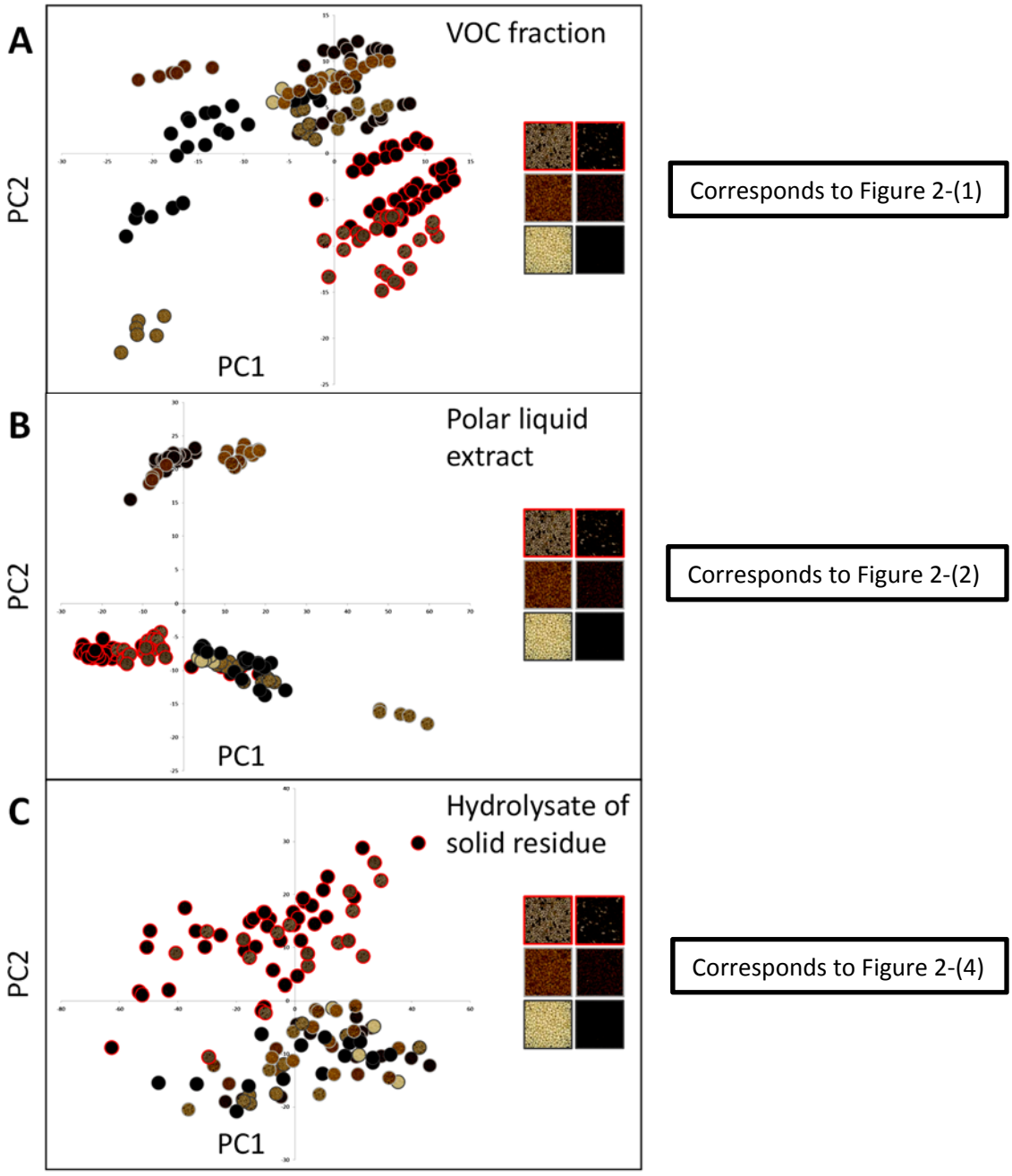


Figure 3. Principal component analyses (PCA) of non-targeted metabolite profiles from VOC, profiling of seed material (A), metabolite profiling of predominantly polar liquid extracts (B) and hydrolysates for solid residue after polar and lipophilic lipid extractions (C). Note that PCA analyses indicate chemical markers that can differentiate chia seeds from linseeds and sesame seeds. Metabolite profiles were performed with multiple technical replicates (n) of each of the 28 seed batches. The first 2 components of each PCA are shown. The percentage of the total variance explained by PC1/PC2 was: 26% (A, n=6); 52% (n=5) and 44% (C, n=2-5), respectively.

From Figure 3 it is evident that the polar liquid extract rendered the best results for discriminating the three studied seeds.

Further analyses were performed to extract relevant and useful chemical markers from each of the data sets. For this purpose, we applied hierarchical cluster analyses, principal component analyses (Figure 3), independent component analyses, for data reduction as well as other statistical analyses, such as 1-way and 2-way analysis of variance (ANOVA), and heteroscedastic Student´s t-testing or Wilcoxon rank sum testing. Finally, we searched for those potential marker molecules with the following properties: (1) non-overlapping standard variations, more stringently (2) with high abundance among chia replicate measurements and distributions that did not overlap with the variation observed in linseed or sesame seed samples, and (3) those chemical markers that were only detectable in chia samples. These logical selection procedures were applied to the initial profiling data sets, which comprise so-called mass features, i.e. sets of defined nominal masses, more exactly m/z ratios, that are reproducibly measured within defined retention time windows. As a initial result we thus obtained mass features that represented candidate marker compounds of seed identity. To identify the marker compounds we matched retention indices (RI) and mass spectra (MS), to the reference data of authenticated compounds from the Golm Metabolome Database (GMD; http://gmd.mpimp-golm.mpg.de/, Kopka et al., 2005) or the NIST collection of mass spectra (http://chemdata.nist.gov/). We assigned identification level S1 if marker candidate and reference compound matched both, by MS and RI. Identification level S2 was assigned if the marker candidate matched by MS only. A third level (S3) was used for those marker candidates without a match. The S3 level indicates that the marker candidate has currently no structural information, but is characterized and can be recognized by chemical property information only. We stored respective MS and RI information of S3 compounds for future identification.

The current status of identified marker candidates is summarized in Table 1, including information on the seed fraction, MS (m/z), RI (n-alkane based retention index) and ranking according to the abundance of the most intense mass feature in arbitrary units, number of co-eluting detectable mass features, current compound annotation and identification level.

Our marker identification is so far most advanced in the predominantly polar liquid extractable fraction. The set of identified compounds indicates that the rosmarinic acid biosynthesis pathway with educts *E*-caffeic acid, catechollactate and the product *E,Z*-rosmarinic acid may be useful markers to monitor chia seeds in rosmarinic acid. In the volatile fraction Caryophyllene appears to be the most important marker to differentiate chia seeds from sesame and linseeds. Besides catechollactate, the hydrolysate of the solid seed residue after both, polar and lipophilic liquid extractions contains several abundant molecules that are indicative of the presence of chia seeds. Identification of these chemicals has been initiated and is currently in progress. These chemicals appear to be hydrolysis resilient compounds with retention and partial mass spectral similarity to

disaccharides. Because these compounds appear to be covalently bound to the seeds we expect that they may originate from the abundant mucilage of chia seeds. However identification of these chemicals is apparently laborious and needs prioritization. We decided that future identification efforts will be prioritized according to the parsimony principle, with focus on one or more of those mass features that will prove to be a relevant chemical marker for monitoring the presence of chia seeds in processed food.

Table 1. Intensity ranked molecular features, nominal mass (m/z) and retention index, with current metabolite identification and metabolomic identification levels (S1-S3).

Nominal mass (m/z)	Retention index	Number of coeluting mass fragments	Average abundance (arbitrary units)	compound name	Identification level	Fraction	Note
205	2640.4	60	71496	-	S3	Hydrolysed solid	Disaccharide
205	2544.0	24	58220	-	S3	Hydrolysed solid	Disaccharide
317	3105.6	4	44445	-	S3	Hydrolysed solid	
375	2604.2	2	39934		S3	Hydrolysed solid	
174	2565.1	20	34156		S3	Hydrolysed solid	
129	2459.4	2	27513		S3	Hydrolysed solid	
275	1804.9	3	25355	citric acid	S1	Hydrolysed solid	
169	2524.3	2	22913		S3	Hydrolysed solid	
247	2559.6	2	13327		S3	Hydrolysed solid	
179	2051.9	2	13250	catechollactate	S2	Hydrolysed solid	
378	2394.7	2	11011		S3	Hydrolysed solid	
229	2413.4	2	10593		S3	Hydrolysed solid	
219	2498.6	1	9258		S3	Hydrolysed solid	
89	2418.1	2	8515		S3	Hydrolysed solid	
396	3399.3	26	54510	*E*-rosmarinic acid	S1	Polar liquid	
267	2051.2	29	15251	catechollactate	S2	Polar liquid	
396	3210.7	34	12463	*Z*-rosmarinic acid	S2	Polar liquid	
219	2133.7	8	5058	*E*-caffeic acid	S1	Polar liquid	
307	2842.3	2	3142	caffeic acid conjugate	S3	Polar liquid	caffeoyl tartaric acid
324	2756.9	11	2495	guanosine	S1	Polar liquid	
249	2834.9	1	1912	ferulic acid conjugate	S3	Polar liquid	feruoyl tartaric acid
249	2696.4	1	826	ferulic acid conjugate	S3	Polar liquid	glucosylferulic acid
161	1558.1	1	801	*E*-cinnamic acid	S1	Polar liquid	
107	2884.8	1	731	-	S3	Polar liquid	
180	1571.8	2	704	2-(4-hydroxyphenyl)ethanol	S1	Polar liquid	
267	1507.8	1	662	salicylic acid	S1	Polar liquid	
311	1710.9	1	609	-	S3	Polar liquid	
229	4289.7	1	444	-	S3	Polar liquid	similar to raffinose
297	2689.3	1	429	-	S3	Polar liquid	similar to guaiacylglycerol
93	1563.3	18	6122	α-caryophyllene	S2	VOC	
79	1487.0	1	1784	bourbonene	S2	VOC	
149	1530.5	7	1480	β-caryophyllene	S2	VOC	
79	1752.1	18	1009	caryophyllene oxide	S2	VOC	
74	480.7	1	715	acetic acid methyl ester	S2	VOC	
93	1736.7	1	342	-	S3	VOC	similar to longipinocarveol
91	1726.7	1	310	-	S3	VOC	similar to stigmastane
161	1761.6	1	278	-	S3	VOC	

2.2 Chemical fractionation and profiling of seeds, and bakery products containing seeds, for the discovery of potential chemical markers by LC-MS (CONICET-ICYTAC).

2.2.1 Seed sampling, extraction and analytical procedures (AR market).

We collected a sample set of chia (*Salvia hispanica* L.), flax (linseed; *Linum usitatissimum* L.) and sesame (*Sesamum indicum* L.), including seed batches that were sold for human consumption in local grocery stores (Córdoba, Argentina). Three different samples of each seed were processed from Argentina (n=9).

Partially defatted chia, sesame and flax flour were obtained according to the process described by Martínez et al. (2012). Briefly, seeds were hydrated to 9.5% moisture, packed in air-tight bags, and stored for 48 h. The bags were shaken regularly to homogenize sample moisture. Hydrated seeds were conditioned to 60°C and pressed using a screw press Komet (Model CA 59 G, IBG Monforts, Germany). Screw speed was 20 rpm. A 5 mm of restriction die was used. The meal obtained after oil extraction was subsequently ground with a coffee mill and passed through a 0.25 mm sieve. This milled fraction represents the defatted seeds used for further analysis.

Seeds and bakery products containing different seed percentages were subjected to targeted metabolomic analysis by UPLC-MS/MS. We focused our study on the polyphenol profile, since these compounds are nutritionally relevant (*e.g.* valuable antioxidant), this being one of the main reasons for using these seeds in processed foods.

For compound extraction, samples were grounded using a laboratory grinder until a fine powder was obtained. A portion of 1 g of the treated sample was extracted with 5 mL of a mixture of methanol/water 50:50. The obtained homogenate was sonicated for 15 minutes, and then centrifuged at 3000 x g for 10 min. The supernatant was separated and the solid pellet re-extracted with 5 mL of the mixture as previously described. These steps were repeated so that the final volume of extract was 20 mL. The combined extracts were filtered, fractionated in Eppendorf tubes and stored at -80°C until analysis. The extraction procedure was carried out in duplicate for each sample.

Compounds were identified and quantified by liquid chromatography coupled with time of flight mass spectrometry detection (HPLC-ESI-Q/TOF). The identification was done by exact mass comparison, $HRMS^n$ and UV-Vis spectra. Quantification was performed by external calibration curves using structurally related compounds.

2.2.2 Chemical markers (polyphenols) in seeds (evaluated by LC-MS):

Table 2 shows compounds identified in three studied seeds, including identification parameters used in each case. Sixty four compounds were identified, including one organic acid, one amino acid, one dihydrochalcone, one dihydroflavonol, 2 flavonones, 3 flavones, 15 flavonols, 15 hydroxycinnamic acids and 25 lignans. **Table 3** shows the quantification of these compounds in partially defatted chia, sesame and flax flours.

As it can be observed, we detected 29 compounds in chia seeds, most of them structurally related to hydroxycinnamic acids. Rosmaric acid and salviaflaside were the most important ones. In the case of sesame, 28 compounds were detected, most of them belonging to the family of lignans, for example matairesinol diglucoside and sesaminol diglucoside to name the most important ones. Finally, we only

detected 10 compounds in flax seeds, which are characterized by eriodictyol-7-O-glucoside.

Table 2. Compounds (markers) identified in chia, flax and sesame seeds.

N°	Compound Name	Molecular Formula	Rt (min)	[M-H]- (m/z) exp.	[M-H]- (m/z) cal.	Error (ppm)	Fragmentation Pattern	Family	Content in seeds
1	**Quinic acid**	$C_7H_{12}O_6$	7.8	191.0561	191.0572	-5.9		Organic Acid	chia
2	**3,5-Dicaffeoylquinic acid**	$C_{25}H_{24}O_{12}$	10.6	515.1335	515.1195	-25.6	353; 191	Hydroxycinnamic acid	flax
3	**Danshesu**	$C_9H_{10}O_5$	11.8	197.0455	197.0449	3.1	179	Hydroxycinnamic acid	chia
4	**Caftaric acid**	$C_{13}H_{12}O_9$	12.2	311.0409	311.038	6.9	179	Hidroxycinnamic acid	chia
5	**Quercetin 3-O-glucosyl-rhamnosyl-glucoside**	$C_{33}H_{40}O_{21}$	12.4	771.1989	771.1989	0	625; 447; 301; 179	Flavonol	chia
6	**Tryptophan**	$C_{11}H_{12}N_2O_2$	12.5	203.0841	203.0826	7.5	186	Aminoacid	sesame, flax, chia
7	**Apigenin 6,8-di-C-glucoside (Vicenin 2)**	$C_{27}H_{30}O_{15}$	12.7	593.1477	593.1512	-5.8	473; 383; 353	Flavone	flax
8	**Nortracheloside**	$C_{26}H_{32}O_{12}$	12.9	535.1827	535.1821	-1.1	373	Lignan	sesame
9	**Caffeic acid hexoside**	$C_{18}H_{18}O_9$	13	341.0878	341.0866	3.4	179	Hydroxycinnamic acid	chia
10	**Salvianolic acid I/H**	$C_{27}H_{22}O_{12}$	13	537.1038	537.1032	-1.2	339; 295	Hydroxycinnamic acid	chia
11	**Myricetin diglucoside**	$C_{27}H_{30}O_{18}$	13.2	641.1359	641.1386	4.2	479	Flavonol	chia
12	**Fertaric acid**	$C_{14}H_{14}O_9$	13.3	325.0565	325.0569	1.1	193	Hydroxycinnamic acid	chia
13	**Eriodictyol-7-O-glucoside**	$C_{21}H_{22}O_{11}$	13.5	449.1116	449.1089	-5.8	287; 269; 259	Flavanone	flax
14	**Nortrachelogenin**	$C_{20}H_{22}O_7$	13.5	373.1281	373.1293	-11.7	355; 327	Lignan	sesame

15	**Lariciresinol diglucoside**	$C_{32}H_{44}O_{16}$	13.6	683.2579	683.2557	3.2	521; 359; 329	Lignan	sesame
16	**Trachelogenin diglucoside**	$C_{33}H_{44}O_{17}$	13.9	711.2461	711.2506	-6.3	387; 323	Lignan	sesame
17	**Apigenin 6-C-xyloside-8-C-glucoside (Vicenin 1)**	$C_{26}H_{28}O_{14}$	14.1	563.1343	563.1406	11.3	443; 383; 353	Flavone	flax
18	**Quercetin diglucoside**	$C_{27}H_{30}O_{17}$	14.2	625.1360	625.1410	-8.0	463; 300	Flavonol	chia
19	**Salvianolic acid E/B/L**	$C_{36}H_{30}O_{16}$	14.2	717.1461	717.15	-5.5	519; 339	Hydroxycinnamic acid	chia
20	**Matairesinol diglucoside**	$C_{32}H_{42}O_{16}$	14.2	681.2391	681.2400	1.4	357; 223; 221	Lignan	sesame
21	**Caffeic acid**	$C_9H_8O_4$	14.6	179.034	179.035	-5.3		Hydroxycinnamic acid	chia
22	**Lariciresinol glucoside**	$C_{26}H_{34}O_{11}$	14.9	521.1988	521.2028	7.8	329	Lignan	sesame
23	**Kaempferol 3-O-galactoside**	$C_{21}H_{20}O_{11}$	14.9	447.0913	447.0933	-4.3	285; 255; 227	Flavonol	flax
24	**Ferulic Acid**	$C_{10}H_{10}O_4$	15.3	193.0490	193.0506	8.6	149	Hydroxycinnamic acid	sesame
25	**Pinoresinol diglucoside**	$C_{32}H_{42}O_{16}$	15.4	681.2364	681.2400	-5.3	357; 323; 151	Lignan	sesame
26	**Taxifolin 3'-glucoside**	$C_{21}H_{22}O_{12}$	15.6	465.1014	465.1038	5.2	303; 202	Dihydroflavonol	flax
27	**Kaempferol diglucoside**	$C_{27}H_{30}O_{16}$	16	609.1461	609.1454	1.2	285	Flavonol	chia
28	**Myricetin glucoside**	$C_{21}H_{20}O_{13}$	16.1	479.0834	479.0831	-0.6	317; 169	Flavonol	chia
29	**Mata/Pino (acetyl)-diglucoside**	$C_{34}H_{44}O_{17}$	16.3	723.2519	723.2506	1.8	519; 357	Lignan	sesame

30	**Mata/Pino Glucoside**	$C_{26}H_{32}O_{11}$	16.4	519.1854	519.1872	-3.5	357	Lignan	sesame
31	**Phloretin-3',5'-di-C-glucoside**	$C_{27}H_{34}O_{15}$	17.1	597.1825	597.1825	0	417; 387; 357	Dihydrochalcone	flax
32	**7-hydroxymatairesinol**	$C_{20}H_{22}O_7$	17.1	373.1301	373.1293	-2.1	355; 311	Lignan	sesame
33	**Salviaflaside (Rosmarinic acid hexoside)**	$C_{24}H_{26}O_{13}$	17.3	521.1301	521.1382	15.6	359; 197	Hydroxycinnamic acid	chia
34	**Trachelogenin glucoside**	$C_{27}H34O12$	17.3	549.1953	549.1978	4.6	387	Lignan	sesame
35	**Mata/Pino Glucoside (isomer)**	$C_{26}H_{32}O_{11}$	17.8	519.1813	519.1872	-11.4	357	Lignan	sesame
36	**Salviaflaside (Rosmarinic acid hexoside)**	$C_{24}H_{26}O_{13}$	18.2	521.1301	521.134	7.5	359; 197	Hydroxycinnamic acid	chia
37	**Myricetin glucoside**	$C_{21}H_{20}O_{13}$	18.5	479.0834	479.0831	-0.6	317; 169	Flavonol	chia
38	**Mata/Pino (acetyl)-glucoside**	$C_{34}H_{43}O_{17}$	18.6	561.1983	561.1978	0.9	357	Lignan	sesame
39	**Quercetin hexoside**	$C_{21}H_{20}O_{12}$	19	463.0882	463.0904	4.7	300	Flavonol	chia
40	**Rosmarinic acid**	$C_{18}H_{16}O_8$	19.1	359.0772	359.0778	1.6	197; 179	Hydroxycinnamic acid	chia
41	**Sesaminol triglucoside**	$C_{38}H_{48}O_{22}$	19.5	855.2728	855.2564	-19.1	-	Lignan	sesame
42	**Rosmarinic acid**	$C_{15}H_{16}O_8$	19.6	359.0785	359.0772	-3.6	197; 179; 161	Hydroxycinnamic acid	chia
43	**Narirutin glucoside**	$C_{33}H_{42}O_{19}$	19.8	741.2248	741.1491	23.2	579	Flavanone	chia
44	**Quercetin hexoside**	$C_{21}H_{20}O_{12}$	20.4	463.0882	463.0906	-5.1	301	Flavonol	chia

45	**Quercetin rhamnoside**	$C_{21}H_{20}O_{11}$	20.8	447.0933	447.0963	6.7	301	Flavonol	chia
46	**Myrcetin**	$C_{15}H_{10}O_8$	21.2	317.0303	317.0303	0	179	Flavonol	chia
47	**Salvianolic acid C**	$C_{26}H_{20}O_{10}$	21.6	491.0984	491.1004	4.2	311; 293	Hydroxycinnamic acid	chia
48	**Matairesinol/Pinoresinol**	C20H22O6	22.1	357.1337	357.1344	1.9	342	Lignan	sesame
49	**Sesaminol Diglucoside (isomer)**	$C_{32}H_{38}O_{17}$	22.4	693.2060	693.2036	-3.4		Lignan	sesame
50	**Methyl rosmarinate**	$C_{19}H_{18}O_8$	22.5	373.0929	373.0959	6.3	179	Hydroxycinnamic acid	chia
51	**Matairesinol/Pinoresinol (isomer)**	$C_{20}H_{22}O_6$	22.7	357.1263	357.1344	22.7		Lignan	sesame
52	**Sesamolinol diglucoside**	$C_{32}H_{40}O_{17}$	22.9	695.2279	695.2193	-12.4		Lignan	sesame
53	**Sesaminol diglucoside (isomer)**	$C_{32}H_{38}O_{17}$	23,3	693,1955	693,2036	11,7	729; 369	Lignan	sesame
54	**Sesaminol diglucoside (isomer)**	$C_{32}H_{38}O_{17}$	23,9	693,1963	693,2036	-10,3		Lignan	sesame
55	**Quercetin**	$C_{15}H_{10}O_7$	24,1	301,0357	301,0354	1.1	273, 229	Flavonol	flax, chia
56	**Sesamolinol glucoside**	$C_{26}H_{30}O_{12}$	24,9	533,1628	533,1664	-6,8	371	Lignan	sesame
57	**Kaempferol rhamnose**	$C_{21}H_{20}O_{10}$	25,1	431,0984	431,1026	9,8	285	Flavonol	chia
58	**Methyl quercetin**	$C_{16}H_{12}O_7$	25,3	315,051	315,0474	-11,4	300	Flavonol	chia
59	**Sesamolinol acetyl-glucoside**	$C_{28}H_{32}O_{13}$	25,6	575,1775	575,1770	-0,8	371	Lignan	sesame

60	**Sesaminol glucoside**	$C_{26}H_{28}O_{12}$	26,1	531,1498	531,1508	-2.0	369	Lignan	sesame
61	**Kaempferol**	$C_{15}H_{10}O_6$	26,9	285,0405	285,0393	-4,2	255, 227	Flavonol	flax, chia
62	**Sesaminol acetyl-glucoside**	$C_{28}H_{30}O_{13}$	27,3	573,1596	573,1614	-3	369	Lignan	sesame
63	**Apigenin**	$C_{15}H_{10}O_5$	27,6	269,0427	269,0455	-10,7	225	Flavone	sesame
64	**Sesaminol**	$C_{20}H_{18}O_7$	32	369,1019	369,0980	-10,5	269	Lignan	sesame

Table 3. Quantification of compounds identified in chia, flax and sesame seeds (µg/g of partially defatted flour). <LOD: below limit of detection.

N°	Compound Name	Standard used for qunatification	CHIA	FLAX	SESAME
1	**Quinic acid**	Quinic acid	60 ± 9	<LOD	<LOD
2	**3,5-Dicaffeoylquinic acid**	Quinic acid	<LOD	1.77 ± 0.01	<LOD
3	**Danshesu**	Caffeic acid	22 ± 2	<LOD	<LOD
4	**Caftaric acid**	Caffeic acid	163 ± 34	<LOD	<LOD
5	**Quercetin 3-O-glucosyl-rhamnosyl-glucoside**	Quercetin	0.27 ± 0.19	<LOD	<LOD
6	**Tryptophan**	Ferulic acid	308 ± 45	691 ± 49	702 ± 23
7	**Apigenin 6,8-di-C-glucoside (Vicenin 2)**	Catechin	<LOD	1.94 ±0.32	<LOD
8	**Nortracheloside**	Catechin	<LOD	<LOD	7.33 ± 0.03
9	**Caffeic acid hexoside**	Caffeic acid	10 ± 3	<LOD	<LOD
10	**Salvianolic acid I/H**	Rosmarinc acid	32 ± 12	<LOD	<LOD
11	**Myricetin diglucoside**	Quercetin	4.4 ± 1.2	<LOD	<LOD
12	**Fertaric acid**	Ferulic acid	281 ± 58	<LOD	<LOD
13	**Eriodictyol-7-O-glucoside**	Quercetin	<LOD	4.6 ± 0.2	<LOD
14	**Nortrachelogenin**	Catechin	<LOD	<LOD	2.0 ± 0.2
15	**Lariciresinol diglucoside**	Catechin	<LOD	<LOD	5.29 ± 0.01
16	**Trachelogenin diglucoside**	Catechin	<LOD	<LOD	1.2 ± 0.1
17	**Apigenin 6-C-xyloside-8-C-glucoside (Vicenin 1)**	Catechin	<LOD	0.5 ± 0.1	<LOD
18	**Quercetin diglucoside**	Quercetin	10.5 ± 1.3	<LOD	<LOD
19	**Salvianolic acid E/B/L**	Rosmarinc acid	144 ± 19	<LOD	<LOD
20	**Matairesinol diglucoside**	Catechin	<LOD	<LOD	127 ±4
21	**Caffeic acid**	Caffeic Acid	9.6 ± 2.4	<LOD	<LOD
22	**Lariciresinol glucoside**	Catechin	<LOD	<LOD	2.6 ± 0.2
23	**Kaempferol 3-O-galactoside**	Kaempferol	<LOD	0.25 ± 0.14	<LOD
24	**Ferulic Acid**	Ferulic acid	<LOD	<LOD	2.7 ± 0.4

25	**Pinoresinol diglucoside**	Catechin	<LOD	<LOD	9.9 ± 0.6
26	**Taxifolin 3'-glucoside**		<LOD	0.30 ± 0.04	<LOD
27	**Kaempferol diglucoside**	Kaempferol	0.7 ± 0.2	<LOD	<LOD
28	**Myricetin glucoside**	Quercetin	13 ± 2	<LOD	<LOD
29	**Mata/Pino (acetyl)-diglucoside**	Catechin	<LOD	<LOD	17.5 ± 0.2
30	**Mata/Pino glucoside**	Catechin	<LOD	<LOD	19.8 ± 0.1
31	**Phloretin-3',5'-di-C-glucoside**	Catechin	<LOD	0.36 ± 0.04	<LOD
32	**7-hydroxymatairesinol**	Catechin	<LOD	<LOD	48 ± 3
33	**Salviaflaside (Rosmarinic acid hexoside)**	Rosmarinic acid	948 ± 145	<LOD	<LOD
34	**Trachelogenin glucoside**	Catechin	<LOD	<LOD	1.5 ± 0.2
35	**Mata/Pino glucoside (isomer)**	Catechin	<LOD	<LOD	1.36 ± 0.08
36	**Salviaflaside (Rosmarinic acid hexoside)**	Rosmarinic acid	200 ± 73	<LOD	<LOD
37	**Myricetin glucoside**	Quercetin	2 ± 1	<LOD	<LOD
38	**Mata/Pino (acetyl)-glucoside**	Catechin	<LOD	<LOD	2.78 ± 0.05
39	**Quercetin hexoside**	Quercetin	6.0 ± 0.7	<LOD	<LOD
40	**Rosmarinic acid**	Rosmarinic acid	46 ± 8	<LOD	<LOD
41	**Sesaminol triglucoside**	Catechin	<LOD	<LOD	12.1 ± 0.1
42	**Rosmarinic acid**	Rosmarinic acid	740 ± 52	<LOD	<LOD
43	**Narirutin glucoside**	Catechin	2 ± 1	<LOD	<LOD
44	**Quercetin hexoside**	Quercetin	0.79 ± 0.08	<LOD	<LOD
45	**Quercetin rhamnoside**	Quercetin	0.75 ± 0.07	<LOD	<LOD
46	**Myrcetin**	Quercetin	17 ± 8	<LOD	<LOD
47	**Salvianolic acid C**	Rosmarinic acid	41 ± 13	<LOD	<LOD
48	**Matairesinol/Pinoresinol**	Catechin	<LOD	<LOD	5.2 ± 0.7
49	**Sesaminol diglucoside (isomer)**	Catechin	<LOD	<LOD	21 ± 2
50	**Methyl rosmarinate**	Rosmarinic acid	36 ± 3	<LOD	<LOD

51	**Matairesinol/Pinoresinol (isomer)**	Catechin	<LOD	<LOD	2.8 ± 0.7
52	**Sesamolinol diglucoside**	Catechin	<LOD	<LOD	51 ± 4
53	**Sesaminol diglucoside (isomer)**	Catechin	<LOD	<LOD	120 ± 8
54	**Sesaminol diglucoside (isomer)**	Catechin	<LOD	<LOD	124 ± 3
55	**Quercetin**	Quercetin	63 ± 37	0.63 ± 0.25	<LOD
56	**Sesamolinol glucoside**	Catechin	<LOD	<LOD	1.1 ± 0.2
57	**Kaempferol rhamnose**	Kaempferol	0.6 ± 0.1	<LOD	<LOD
58	**Methyl quercetin**	Quercetin	0.3 ± 0.1	<LOD	<LOD
59	**Sesamolinol acetyl-glucoside**	Catechin	<LOD	<LOD	7.74 ± 0.08
60	**Sesaminol glucoside**	Catechin	<LOD	<LOD	4.6 ± 0.5
61	**Kaempferol**	Kaempferol	2.39 ± 0.03	0.06 ± 0.04	<LOD
62	**Sesaminol acetyl-glucoside**	Catechin	<LOD	<LOD	2.95 ± 0.06
63	**Apigenin**	Catechin	<LOD	<LOD	4.0 ± 0.6
64	**Sesaminol**	Catechin	<LOD	<LOD	51.6 ± 0.4

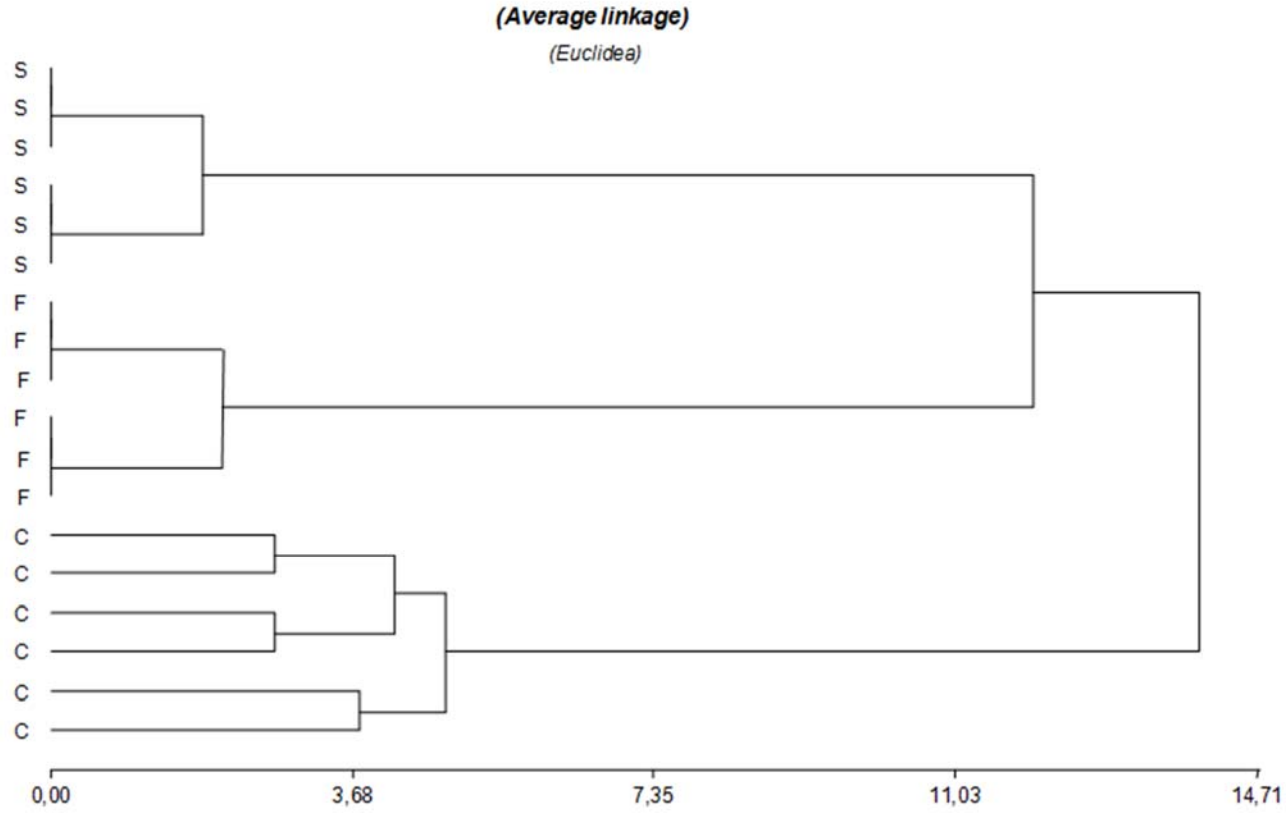
2.2.3 Evaluation of chemical markers in seeds by LC-MS and chemometrics:

To explore and classify the different seeds samples, Cluster Analysis (CA) and Principal Component Analysis (PCA) were performed. These chemometric methods for data mining also allow extraction of marker candidates for the studied seeds.

2.2.3.1 Cluster Analysis (CA).

CA, using the concentration of the 64 compounds identified, was carried out as a first exploratory method for the evaluation of the data structure. The amalgamation criterion to CA was average linkage, and the distance method was Euclidean. Results obtained are presented as a dendrogram, which is shown in **Figure 4**, where we can observe that the sample arrangement clearly separated three clusters, corresponding to sesame, flax and chia seeds, at a Euclidean distance of 5.

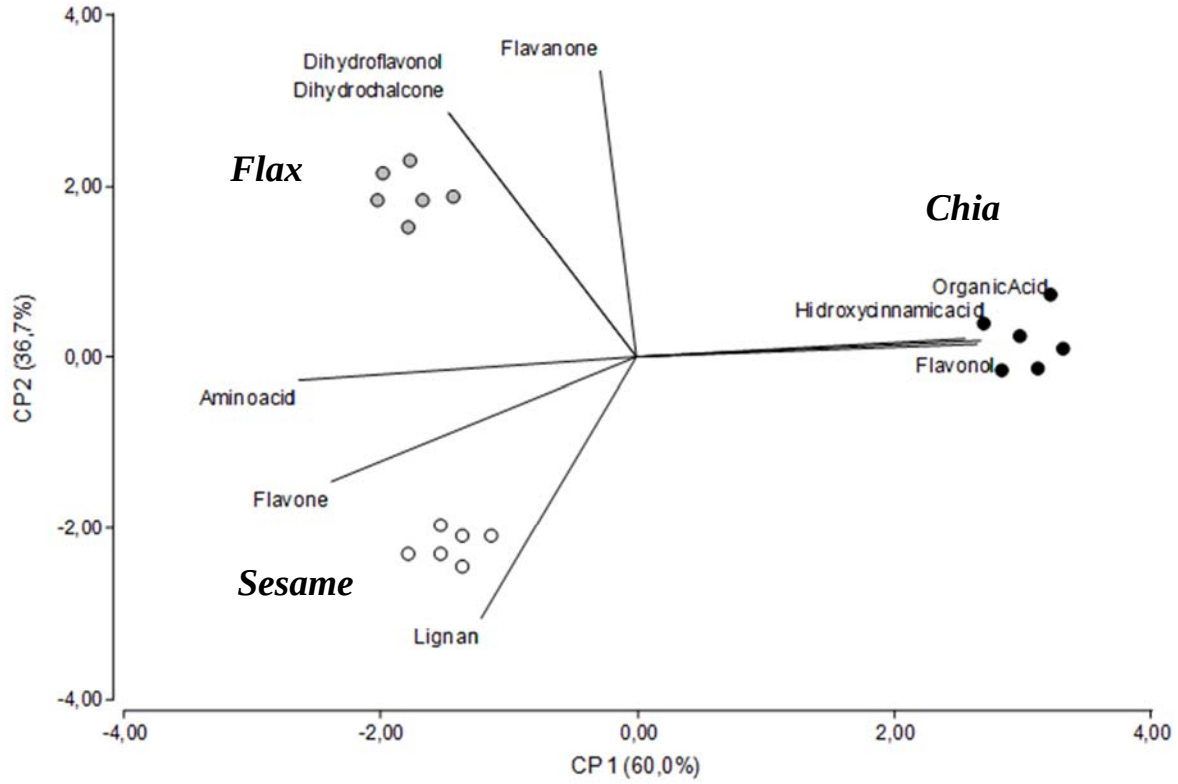
Figure 4. Dendrogram of chia, flax and sesame samples analysed by LC-MS. S: sesame; F: flax and C: chia.



2.2.3.2 Principal Components Analysis (PCA).

For PCA analysis the same set of quantified compounds was clustered according to their secondary metabolite family (as shown in **Table 2**). The PCA model was obtained using two principal components (PCs), which explained 96.7% of the variance. PC1 accounts for 60% of the variance and was mainly characterized by the organic acid, the amino acid and hydroxycinnamic acids. PC2 contributes to 36.7% of the variance and was mainly characterized by lignans and flavanones. The scatter plot obtained from PCA scores is illustrated in **Figure 5**, showing that samples are separated into three well defined groups, each corresponding to one out of the three studied seeds.

Figure 5. Principal components (PCA) plot of chia, flax and sesame samples analysed by LC-MS.

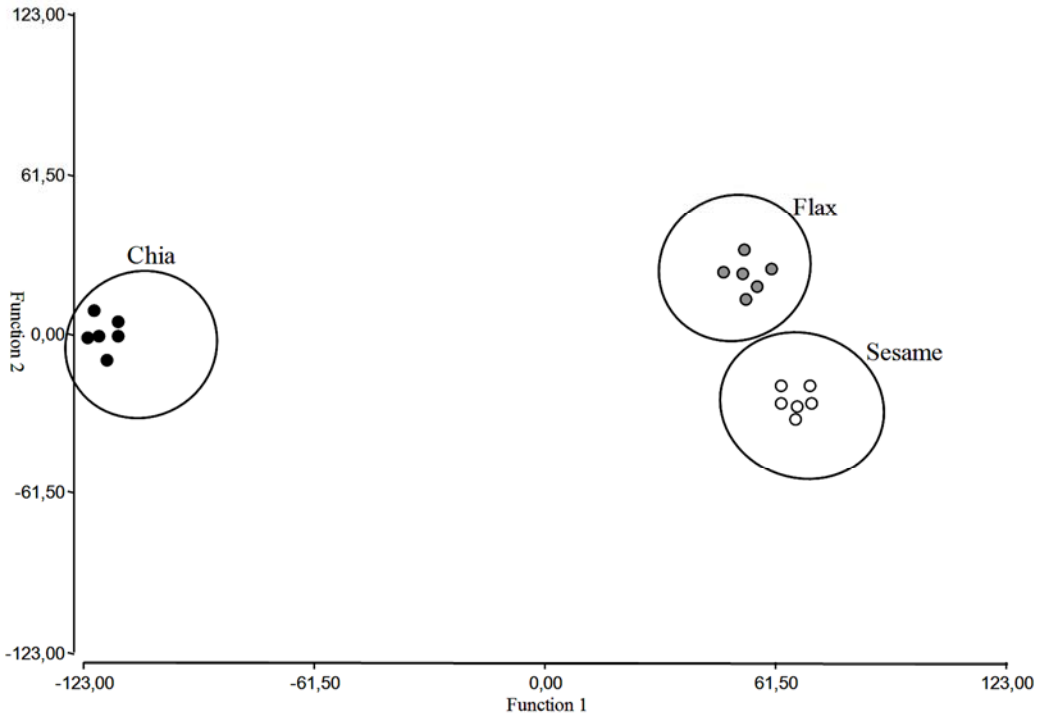


2.2.3.3 Discriminant Analysis (DA).

DA was performed to evaluate if seeds samples could be mathematically distinguished according to their polyphenolic profile and clustered according their chemical family. The robustness of the classification model was evaluated by a cross-validation test, using the "leave-one-out" procedure.

The application of DA allowed predicting the origin of samples with 100% accuracy using two independent discriminant functions (**Figure 6**). The most important variables in the discrimination were the content of hydroxycinnamic acids, flavonols and lignans.

Figure 6. Scatter plot of two discriminant functions showing separation between chia, flax and sesame samples.



Considering LC-MS results, we suggest the use of hydroxycinnamic acids, flavonols and lignans to differentiate between studied seeds, using these compounds as markers of authenticity in complex foods containing these seeds. Samples from different geographical origin need to be evaluated to confirm this suggestion. Further work is in progress to exchange seed samples between MPIMP and ICYTAC to validate the proposed chemical markers (deliverable 14.2).

2.3 Preparation and analysis of bakery products containing studied seeds:

When proposing chemical markers for food products, it is important to evaluate their stability after food processing. Thus, we prepared bakery goods (sweet cookies) partially replacing wheat flour with the partial defatted flour prepared from each studied seed. Additionally, bakery goods were also prepared using wheat flour with the addition of whole seeds. Furthermore, we also prepared fresh pasta with a partial replacement of wheat flour by the partial defatted flour of chia, looking to verify the stability of proposed chemical markers along the food production.

2.3.1 Preparation of Baked goods (cookies).

Cookies were produced with the defatted flours of the seeds or with whole chia, flax and sesame seeds.

Cookies prepared with defatted flour: For each seed, three formulations containing 5, 10 and 20% of defatted "seed flour" was used to replace the equivalent amount of wheat flour. A control formulation using only wheat flour was also produced.

Firstly, wheat flour (45g) and the corresponding amount of defatted seed flour (2.25g, 4.5g or 9g) were mixed (Mix 1). Separately, fat (20.2g) and powdered sugar (27g) were mixed with an electric mixer for 3 minutes (Mix 2). In another container, the additives: salt (0.42g), sodium bicarbonate (0.5g) and skimmed powdered milk (2.25g) were added (Mix 3). Then Mix 2 and Mix 3 were combined and stirred for 1 min using an electric mixer. Then, water (4mL) was added to the combined Mix 2 + Mix 3, mixing for 1 min. Finally, Mix 1 was added and stirred for 2 min until a soft dough was obtained.

The dough was stretched with a rolling pin and laminated to obtain a uniform dough (0.8 cm height). After that, cookies were made using a metallic cutter (internal diameter 45 mm). The cookies were placed on a greaseproof paper and distributed on a plate. Afterwards, cookies were baked for 11 min at 180°C, yielding 4 cookies.

Cookies prepared with whole seeds: Cookie were produced with whole chia, flax or sesame seeds. For each seed, two formulations containing 10 and 20% whole seeds were incorporated to the total content of wheat flour. A control formulation without added seeds was also produced. Furthermore, two formulations containing all three studied seeds in equal parts (10 and 20%) were also prepared.

Firstly, wheat flour (45g) was mixed with the corresponding amount of whole individual seeds (4.5g or 9g when only individual seeds were tested; 1.5g or 3 g of each seed when preparing three-seeds cookies) (Mix 4). Mix 2 and Mix 3 were prepared as previously described. Then Mix 2 and Mix 3 were combined and stirred for 1 min using an electric mixer. Then, water (4mL) was added to the combined Mix 2 + Mix 3, mixing for 1 min. Finally, Mix 4 was added and stirred for 2 min until a soft dough was obtained.

The dough was stretched with a rolling pin and laminated to obtain a uniform dough (0.8 cm height). After that, cookies were made using a metallic cutter (internal diameter 45 mm). The cookies were placed on a greaseproof paper and distributed on a plate. Afterwards, cookies were baked for 11 min at 180°C, yielding 4 cookies.

2.3.2 Preparation of pasta supplemented with defatted chia flour.

A small-scale standardized laboratory procedure was used for pasta manufacture. Pasta was prepared with different concentrations of partially defatted chia flour (0, 2.5, 5.0, and 10%, respectively, weight on wheat flour basis). For each formulation pasta flour, water, and salt (50g, 22.5g, and 1.0g, respectively) were mixed in a Hobart bench top mixer (Hobart Inc., Troy, OH, USA) until the dough had an adequate consistency for lamination. Dough was divided by hand in appropriate size and was laminated using a pasta home-scale size lamination machine (Drago, Inc., China), using a 3-step procedure: hand lamination, up to approximately 10-mm thickness; roll lamination, up to a 5-mm thickness; and final roll laminate onto a 2-mm thickness (final pasta thickness). Laminated pasta sheets were cut using a cutting roll (2-mm wide) obtaining the pasta strings (2 x 2 x 200 mm). Pasta strings were suspended in wooden sticks on a wooden rack. Pasta was dried using a two-stage process: pre-drying at 30°C for 30 minutes (with forced air circulation), followed by 24 h at 30°C in a closed chamber (relative humidity 70%). Dried pasta was stored in airtight bags at room temperature until analysis.

2.3.3 Evaluation of the stability of proposed chemical markers in products.

Polyphenols were extracted from cookies with the same protocol described in **2.2.1**.

Polyphenols were extracted from different pasta as follows: Dried pasta samples were ground using a coffee grinder. In parallel, an independent batch of dried pasta samples were cooked in ultra-pure water at their respective OCT (Optimum Cooking Time). Afterwards, cooked pasta was lyophilized and ground. Five grams of either uncooked pasta powder or lyophilized cooked pasta powder were extracted with 20mL of a mixture acetone/water (4:1), for 1h at room temperature in darkness.

Both cookies and pasta extracts were analysed by LC-MS as described in **2.2.2**, looking to evaluate the stability of their polyphenol profile after cooking.

2.3.3.1 Polyphenol profile of cookies supplemented with defatted chia flour and whole chia seeds.

Twelve out of 29 compounds detected in chia seeds were detected in cookies supplemented with defatted chia flour. Furthermore, 11 out of starting 29 polyphenols were found in cookies prepared with addition of whole chia seeds. In both cases, most of the detected compounds are hydroxycinnamic acids, derived from rosmarinic acid. Quantification of these polyphenols is shown in **Table 4**. In most cases, there is a positive correlation between the concentration of each compound found in the cookies with the percentage of chia flour or whole seeds added. *Rosmarinic acid* and *Salviaflaside* were the compounds found in higher concentration. These two compounds seem to be the more stable throughout the preparation and cooking procedures applied.

Table 4. Quantification of polyphenols in cookies supplemented with chia (µg/g cookie).

Compound	Partially defatted chia flour added			Whole chia seeds added	
	Cookie Chia 20%	Cookie Chia 10%	Cookie Chia 5%	Cookie Chia 20%	Cookie Chia 10%
Quinic acid	14 ± 5	12 ± 5	4 ± 3	16 ± 2	12 ± 1
Danshesu	<LOD	<LOD	<LOD	2.7 ± 0.6	1.3 ± 0.5
Caftaric acid	13 ± 8	<LOD	<LOD	0.68 ± 0.02	<LOD
Quercetin 3-O-glucosyl-rhamnosyl-glucoside	0.12 ± 0.09	<LOD	<LOD	<LOD	<LOD
Caffeic acid hexoside	<LOD	<LOD	<LOD	165 ± 49	150 ±14
Salvianolic acid I/H	<LOD	<LOD	<LOD	1.62 ± 0.44	<LOD
Quercetin diglucoside	3 ± 1	1.2 ± 0.4	0.5 ± 0.2	<LOD	<LOD
Salvianolic acid E/B/L	26 ± 7	1.8 ± 0.1	2.1 ± 0.5	44 ± 7	13 ± 5
Myricetin glucoside	2.1 ± 0.2	<LOD	<LOD	<LOD	<LOD
Salviaflaside	108 ± 10	55 ± 14	30 ± 7	27 ± 12	12 ± 7
Salviaflaside (Isomer)	34.1 ± 0.5	15 ± 2	2.92 ± 0.01	19 ± 8	12 ± 6

Quercetin hexoside	0.88 ± 0.07	0.6 ± 0.1	<LOD	<LOD	<LOD
Rosmarinic Acid	109 ± 10	56 ± 1	17 ± 5	52 ± 11	17.1 ± 0.3
Rosmarinic acid (isomer)	<LOD	<LOD	<LOD	0.34 ± 0.05	<LOQ
Salvianolic acid C	5.8 ± 0.7	<LOD	<LOD	2.8 ± 0.2	0.23 ± 0.09
Methyl rosmarinate	3 ± 3	<LOD	<LOD	<LOD	<LOD

2.3.3.2 Polyphenol profile of cookies supplemented with defatted sesame flour and whole sesame seeds.

Fifteen out of 28 compounds detected in sesame seeds were detected in cookies supplemented with defatted sesame flour. Furthermore, 11 out of starting 28 polyphenols were detected in cookies with whole sesame seeds added. In both cases, most of the compounds detected are structurally related to lignans. Quantification of these polyphenols is shown in **Table 5**. The addition of the partially defatted sesame flour to cookies, instead of whole seeds, allowed a better extraction of the compounds of interest. The concentration of most compounds found showed a positive correlation with the percentage of sesame flour added to the cookies. *Matairesinol diglucoside*, *Sesamolinol diglucoside* and *Sesaminol diglucoside* were the compounds found in higher concentration in all types of sesame cookies. Therefore, these compounds could be pointed out as the most stable throughout the cooking procedure applied.

Table 5. Quantification of compounds in cookies supplemented with sesame (µg/g cookie).

	Partially defatted sesame flour added			**Whole sesame seeds added**	
Compounds	**Cookie sesame 20%**	**Cookie sesame 10%**	**Cookie sesame 5%**	**Cookie sesame 20%**	**Cookie sesame 10%**
Nortracheloside	<LOD	<LOD	<LOD	0.47 ± 0.04	0.16 ± 0.04
Nortrachelogenin	<LOD	<LOD	<LOD	0.35 ± 0.06	0.19 ± 0.09
Lariciresinol diglucoside	0.60 ± 0.20	0.23 ± 0.01	0.07 ± 0.01	<LOD	<LOD
Trachelogenin Diglucoside	<LOD	<LOD	<LOD	0.56 ± 0.05	0.24 ± 0.08
Matairesinol diglucoside	27 ±13	10 ± 5	6.1 ± 0.2	10.8 ± 0.1	6.4± 0.4
Lariciresinol glucoside	0.63 ± 0.01	0.16 ± 0.01	0.07 ± 0.01	<LOD	<LOD
Pinoresinol diglucoside	1.1 ± 0.6	0.7 ± 0.5	0.11 ± 0.01	<LOD	<LOD
Mata/Pino (acetyl)-diglucoside	1 ± 1	0.57 ± 0.01	0.29 ± 0.01	0.22 ± 0.04	0.88 ± 0.06
Mata/Pino glucoside	1.9 ± 1.4	0.94 ± 0.01	0.66 ± 0.03	<LOD	<LOD
Sesaminol triglucoside	1.1 ± 0.8	0.5 ±0.5	0.24 ± 0.03	6.4 ± 0.4	4.2 ± 0.1
Matairesinol/Pinoresinol	0.13 ± 0.04	<LOD	<LOD	<LOD	<LOD
Sesaminol diglucoside (isomer)	1.7 ± 0.8	0.8 ± 0.6	0.40 ± 0.07	1.2 ± 0.5	0.6 ± 0.2

2.3.3.3 Polyphenol profile of cookies supplemented with defatted flax flour and whole flax seeds.

Three out of 10 compounds detected in defatted flax flour were detected in cookies supplemented with flax flour. Furthermore, one out of 10 polyphenols were detected in cookies with whole flax seeds added. Quantification of these polyphenols is shown in **Table 6**. The concentration of most compounds found showed a positive correlation with the percentage of flax flour added to the cookies. Apigenin 6-C-xyloside-8-C-glucoside was the compound with higher recovery in flax cookies.

Table 6. Quantification of compounds in cookies supplemented with Flax (µg/g cookie).

Compound	Partially defatted flour added			Whole sedes added	
	Cookie Flax 20%	Cookie Flax 10%	Cookie Flax 5%	Cookie Flax 20%	Cookie Flax 10%
Apigenin 6,8-di-C-glucoside	0.69 ± 0.03	<LOD	<LOD	<LOD	<LOD
Apigenin 6-C-xyloside-8-C-glucoside	1.8 ± 1.1	0.32 ± 0.18	0.50 ± 0.01	1.0 ± 0.3	0.01 ± 0.00
Eriodictyol-7-O-glucoside	0.42 ± 0.25	0.34 ± 0.21	0.23 ± 0.04	1.68 ± 0.40	0.36 ± 0.19

2.3.3.4 Polyphenol profile of cookies supplemented with chia, sesame and flax whole seeds.

Since in the market it is common to find cookies containing the three types of seeds, we prepared cookies supplemented with chia, sesame and flax seeds at two different levels (20 and 10%). Considering that these cookies have the three studied seeds, we performed an additional quantification of polyphenols in the resulting mixes. Thus, only 18 out of 64 compounds originally detected were found in amounts exceeding the quantification limit (LOQ). Six out of these 18 compounds were associated to chia seeds (mainly rosmarinic acid derivatives), 10 corresponded to sesame seeds (most of them lignans) and 2 corresponded to flax seeds (**Table 7**). Thus, compounds of detected in these cookies were characteristic of the original seeds, being the same detected in cookies prepared from single seeds. Therefore, these 18 compounds could be pointed out as chemical markers of the three studied seeds.

Table 7. Polyphenols quantified in cookies supplemented with chia, sesame and flax whole seeds (µg/g cookie).

Compound	Seed mix 20%	Seed mix 10%
Quinic acid	9.2 ± 0.1	6 ± 1
Caffeic acid hexoside	165 ± 5	136 ± 23
Eriodictyol 7-O-glucoside	0.20 ± 0.09	0.03 ± 0.02
Matairesinol diglucoside	0.24 ± 0.08	<LOD
Matairesinol diglucoside	4.42 ± 0.02	2 ± 1
Salvianolic acid E/B/L	11.6 ± 0.2	6.1 ± 0.2
Apigenin 6-C-xyloside-8-C-glucoside	0.60 ± 0.06	0.45 ± 0.02
Ferulic Acid	2.3 ± 2.6	<LOD
Mata/Pino (acetyl)-diglucoside	0.72 ± 0.09	<LOD
Salviaflaside	37 ± 2	16 ± 3
Salviaflaside (isomer)	4.6 ± 1.2	3.4 ± 1.1
Sesaminol triglucoside	2.43 ± 0.06	1.0 ± 0.1
Rosmarinic acid	13.2 ± 0.7	5.3 ± 1.6
Sesaminol diglucoside	0.32 ± 0.10	0.07 ± 0.05
Sesamolinol diglucoside	5.5 ± 0.3	2.0 ± 0.3
Sesaminol diglucoside (isomer)	35 ± 2	17 ± 3
Sesaminol diglucoside (isomer)	3 ± 3	1.6 ± 0.4
Sesamolinol acetyl-glucoside	0.18 ± 0.04	< LOD

2.3.3.5 Polyphenol profile of pasta supplemented with defatted chia flour.

Twelve out of 29 compounds detected in defatted chia flour were detected in pasta supplemented with chia flour. All of 12 quantified polyphenols showed a positive correlation with the amount of chia flour in both raw (dried) and boiled pasta (**Table 8**). It is worth to remark that the boiling process affected the amount of phenolic compounds in relation to the starting raw pasta (Table 8). Caffeic acid, caffeic acid hexoside and danshensu showed higher concentrations in boiled pasta relative to raw pasta. However, rosmarinic acid glycoside, one of the most abundant components in raw pasta, was not detected after boiling. This drastic change indicates that chemical modifications are occurring during the boiling process, which could explain the observed changes. On the other hand, levels of rosmarinic acid were not significantly affected by the boiling process, contributing to preserve the antioxidant capacity of the chia-supplemented pasta. It is also noteworthy to mention that compounds that were stable during cooking were also stable during pasta manufacturing, with the exception of salviaflaside.

Table 8. Quantification of compounds detected in raw and boiled paste supplemented with chia.

Compound	Raw pasta chia 2.5%	Raw pasta chia 5%	Raw pasta chia 10%	Boiled pasta chia 2.5%	Boiled pasta chia 5%	Boiled pasta chia 10%
Caffeic acid	0.80 ± 0.12	0.95 ± 0.16	1.4 ± 0.1	2.9 ± 0.1	4.3 ± 0.4	5.1 ± 0.2
Quinic acid	1.3 ± 0.1	1.3 ± 0.1	1.53 ± 0.07	0.42 ± 0.01	0.53± 0.03	0.67 ± 0.04
Danshensu	0.47 ± 0.01	0.66 ± 0.04	1.17 ± 0.03	1.5 ± 0.2	2.8 ± 0.2	3.8 ± 0.6
Caftaric acid	<LOD	<LOD	0.51 ± 0.72	<LOD	<LOD	<LOD
Fertaric acid	<LOD	<LOD	3.2 ± 0.1	<LOD	<LOD	<LOD
Caffeic acid hexoside	<LOD	<LOD	0.40 ± 0.56	37.4 ± 3.0	39.0 ± 0.1	38.8 ± 1.4
Rosmarinic acid	41.1 ± 4.4	76 ± 10	130 ±16	36.4 ± 1.6	73.3 ± 6.6	112.8 ± 0.2
Methyl rosmarinate	0.56 ± 0.05	0.49 ± 0.19	0.96 ± 0.07	0.18 ± 0.26	0.75± 0.13	1.34 ± 0.03
Salvianolic acid C	0.56 ± 0.01	0.93± 0.13	1.86 ± 0.25	0.22 ± 0.07	1.40± 0.01	2.30 ± 0.12
Salviaflaside	21.8 ± 3.5	50 ± 6	94 ± 7	<LOD	<LOD	<LOD
Salvianolic acid H/I	<LOD	<LOD	0.53 ± 0.16	0.02 ±0.01	0.05 ±0.01	0.13 ± 0.03
Salvianolic acid E/B/L	0.24 ± 0.03	0.52 ± 0.06	1.02 ± 0.08	<LOD	<LOD	<LOD

3. CONCLUSIONS AND PERSPECTIVES.

- ✓ The analysis of volatile compounds (VOCs) by GC-MS contributes to the identification of raw seeds, presenting characteristic compounds that can be used as chemical markers for seed authentication. Further work is ongoing to verify if VOCs can be also used in processed (cooked or boiled) foods to identify the presence of studied seeds,
- ✓ The analysis of both polar liquid fraction and hydrolyzed solid fraction by GC-MS also provides useful chemical markers characteristics of raw seeds. It is also necessary to verify if these markers remain after food-processing (cooking-boiling).
- ✓ The analysis of polyphenols by LC-MS provides additional and clear evidence on the presence of characteristic compounds, namely chemical markers, for three studied seeds (chia, sesame and flax).
- ✓ Some of these polyphenols were stable to both boiling and cooking processes and were found in final products (cookies and boiled pasta). Thus, the idea of using chemical markers to ensure food integrity remains as a feasible alternative so far.
- ✓ At present samples processed by MPIMP and CONICET-ICYTAC are being exchanged to verify the scope of proposed markers on products from different markets.
- ✓ Future work on standardization of extraction and analytical procedures, including data mining, will be performed to prepare a SOP as part of the next deliverable.

4. References:

Agudelo-Romero P, Erban A, Sousa L, Pais MS, Kopka J, Fortes AM (2013) Search for transcriptional and metabolic markers of grape pre-ripening and ripening and insights into specific aroma development in three Portuguese cultivars. PLoS ONE 8(4): e60422

Dethloff F, Erban A, Orf I, Alpers J, Fehrle I, Beine-Golovchuk O, Schmidt S, Schwachtje J, Kopka J (2014) Profiling methods to identify cold-regulated primary metabolites using gas chromatography coupled to mass spectrometry. In: Hincha DK, Zuther E (eds) Plant cold acclimation: Methods and Protocols. Methods in Molecular Biology 1166: 171-197.

Erban A, Schauer N, Fernie AR, Kopka J (2007) Non-supervised construction and application of mass spectral and retention time index libraries from time-of-flight GC-MS metabolite profiles. In: Weckwerth W (ed) Metabolomics: Methods and protocols. Methods in Molecular Biology 358: 19-38

Fuentes P, Zhou F, Erban A, Karcher D, Kopka J, Bock R (2016) High-level production of the antimalarial drug precursor artemisinic acid in tobacco plants. eLife 5: e13664

Gal A, Wirth R, Kopka J, Fratzl P, Faivre D, Scheffel A (2016) Macromolecular recognition directs calcium ions to coccolith mineralization sites. Science 353(6299): 590-593

Hummel J, Strehmel N, Selbig J, Walther D, Kopka J (2010) Decision tree supported substructure prediction of metabolites from GC-MS profiles. Metabolomics 6: 322-333

Kopka J, Schauer N, Krueger S, Birkemeyer C, Usadel B, Bergmueller E, Doermann P, Weckwerth W, Gibon Y, Stitt M, Willmitzer L, Fernie AR, Steinhauser D (2005) GMD@CSB.DB: the Golm Metabolome Database. Bioinformatics 21 (8): 1635-1638

Martínez, M.L., Marín, M.A., Faller, C.M.S., Revol, J., Penci, M.C. and Ribo, P.D. 2012. Chia (Salvia hispanica L.) oil extraction: Study of processing parameters. LWT - Food Science and Technology 47: 78-82.