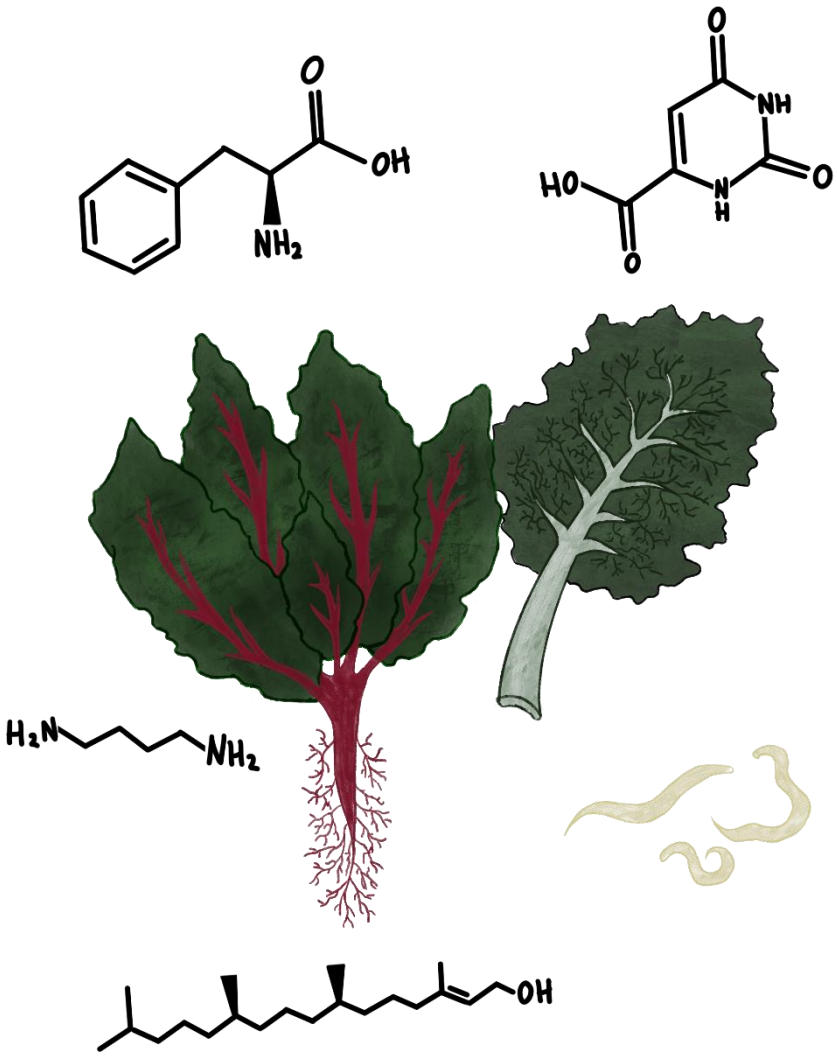
METABOLOMICS ANALYSIS OF CHARDS AND NMR PROFILING OF THE CAENORHABDITIS ELEGANS METABOLOME WITH PARKINSON'S DISEASE



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ABSTRACT

Plants have been demonstrated to be beneficial to health. The omics science has led to perform widely studies of these benefits. The present study aims to find out new compounds and new biological effects of chards. Differences have been shown between different parts of 28 diverse chards (leaf, stem, and root) with possible beneficial biological effects. In addition, a metabolomic study of Caenorhabditis elegans (C. elegans) has been done to see if this is a good differential models for NMR metabolomics studies on Parkinson's disease (PD). With a positive result.

INTRODUCTION

Metabolomics is the study of small molecules called metabolites found in the organism. This is a comprehensive, unbiased, high-throughput study that allows not only the identification of each individual metabolite, but also its quantification (1). Metabolites are the end product of cellular regulation processes, metabolism, gene expression, kinetic activity and enzyme regulation among other processes (2). Furthermore, metabolites are highly variable depending on when and where they occur. So, it can be said that metabolomics in comparison to other omics sciences, such as proteomics and transcriptomics, is closer to the phenotype and function in each organism, tissue, or cell (3,4). Currently, metabolomics is being of great use to interpret pathological states of human diseases such as cancer, diabetes, neurodegenerative diseases, autoimmune diseases, coronary heart disease, etc. It is also being used for biotechnological applications such as food, pharmacology, toxicology, plant biotechnology and others (5). The application of metabolomics in plants started in the early 1990s (6). Plants produce more than 200,000 metabolites, that perform different functions (2). It makes It more challenging to define the metabolome caused by their very complex nature since there is a great deal of chemical structure diversity among the metabolites, as well as a huge diversity of concentrations (5). But in the face of these difficulties, plant metabolomics has recently become one of the main focuses of much research because plants have multiple benefits for human health through their intake or use in drugs, among many other utilities (7).

As mentioned before, metabolomics is being useful to interpret pathological states of certain illness, including neurodegenerative diseases. Among neurodegenerative pathologies, the second most common after Alzheimer's disease is Parkinson's disease (PD), affecting around 10 million people worldwide (8). PD is characterised by severe motor impairment due to a decrease in dopamine, which is attributed to a progressive loss of dopaminergic neurons in the substantia nigra, a region crucial for voluntary motor functions (9).

To study the pathological stages, to find an improved treatment or solution to PD, different experimental models have been used, including *Caenorhabditis elegans* (C. elegans). It is an eukaryotic model of approximately 1 mm, non-pathogenic, in which

hermaphrodites and males exist, the last in a proportion of 0.05%. The advantages of using C. elegans as a research model are several, including the fact that they are very economical, as they have a short and defined life cycle, a high reproductive capacity, and very cheap cost to maintain as they can be grown on E. coli lawn on agar medium (10). In addition, C. elegans has a defined and unchanging nervous system with 302 neurons in each worm and a total of 959 cells (11). Compared to other research models, the connections between neurons have been mapped and they can also be transgenically manipulated to express human genes, making them a very good model for studying diseases, including neurodegenerative diseases such as PD, as they possess the major neurotransmitter systems of dopaminergic neurons (12).

Different equipments are used to perform metabolomics for both plant and C. elegans profiling, the most common are mass spectrometry (MS) or nuclear magnetic resonance spectrometry (NMR) (13). Both techniques require prior extraction of the metabolites, which can lead to loss or degradation of the metabolites. This would be solved by using high-resolution magic angle NMR (HR-MAS) which leaves the sample intact (3).

When you have two types of spectroscopy to use, it is impossible not to compare what is the most accurate platform when performing a metabolomics study . If you are looking for sensitive equipment, you should use MS, as the detection limit goes down to nanomolar, whereas NMR sensitivity is low, although it can be improved by increasing the field strength, using different types of cryoprobes, microprobes and dynamic nuclear polarisation. In selectivity, MS is more useful because it can be used for targeted analysis, while NMR is generally used for non-targeted analysis (3). But in NMR, all metabolites that have a concentration suitable for NMR can be detected in one single measurement, whereas in MS different chromatographic techniques are usually needed for the detection of the different metabolites. In addition, MS is a destructive technique, although it simply requires a small amount of sample, whereas in NMR the sample is easily retrievable and can be stored for a long time (14). Another thing in favour of NMR is that it has a high reproducibility because it requires minimal sample preparation among other analysis, whereas in MS the reproducibility is moderate. This is due to the fact that, among other things, the sample preparation is more demanding, requiring different columns and optimization of ionisation conditions. Furthermore, it should be

added that NMR can be used for in vitro and in vivo studies (15). In conclusion, we can determine that for non-targeted metabolomics analysis would be better to use NMR.

In NMR, molecules are detectable when one or more atoms with a non-zero spin have a magnetic moment. This makes most of biologically important molecules detectable by NMR, because the isotopes with non-zero magnetic moments are ^{1}H, ^{13}C, ^{14}N, ^{15}N and ^{31}P (16). The resulting signal in an NMR spectrum differs from the other signals by its frequency, which is related to its chemical shift, its intensity, and its magnetic relaxation properties. Therefore, as mentioned above, NMR spectra usually provide complete information about the identity of each metabolite in the sample. However, sometimes there are problems, since there can be overlapped signals that limit NMR metabolomic analysis (5).

OBJECTIVES

The main aim was to find new compounds in chard to see new beneficial of consuming chards. Different specific aims were proposed to achieve the main one:

Identification of the metabolites of different chard by NMR and their differentiation between different parts (leaf, stem, and root).

Try to relate the differential metabolites of the different parts of chards to a possible diet-base treatment or the use of chards to create drugs helping to prevent or treat PD. Also, to stablish a model for PD that can be differentiated on NMR.

MATERIALS AND METHODS

Sample preparation (Chard).

In this study, we used 28 different types of chards that were harvest in Munia with different characteristics grown. Each chard was divided in 3 samples, leaf, stem, and root. Chard samples were freeze-dried and powdered as much as possible with a spatula. From here we followed a protocol for the extraction of metabolites in two parts (polar (which we call aqueous) and lipid). This protocol was modified (17). The first step was to measure approximately 15mg of sample (Table 1) in 2mL Eppendorfs®. The aqueous phase of each sample was extracted using an 8:2 ratio of bi-distilled water and methanol, respectively. They were mixed by vortexing and left to rest 15 minutes at room temperature. The samples were centrifuged at 19.000x g for 15 minutes at 4^{o}C, in order to induce the separation of the plant debris. To finish with the aqueous phase, the supernatant, containing the polar metabolites of each sample, were carefully transferred with a pipette to others 2mL Eppendorfs® and dried under nitrogen flow. Using the pellet from the first Eppendorfs®, the lipid extraction of the samples was started by adding a mixture of 800µl of chloroform and 400µl of methanol to each sample, while the samples were on ice to avoid the maximum possible evaporation of the reagents. Then, samples were vortexed and left to rest at room temperature for 10 minutes. The rested samples were centrifuged at 19.000x g for 15 minutes at 4^{o}C . The supernatant (the lipidic phase of each sample) was transferred by pipette to other Eppendorfs® and dried by a flow of nitrogen to keep only the metabolites.

Table 1.- Weight of chards

Nº of chard	Part of the chard	Weight	Nº of chard	Part of the chard	Weight
1	Leaf	14.8	15	Leaf	14.1
1	Stem	15.1	15	Stem	15
1	Root	15.5	15	Root	15
2	Leaf	14.8	16	Leaf	14.5
2	Stem	14.8	16	Stem	15.5
2	Root	14.3	16	Root	15.3
3	Leaf	14.9	17	Leaf	14.8
3	Stem	14.5	17	Stem	15.4
3	Root	15.3	17	Root	16.1
4	Leaf	14.6	18	Leaf	15
4	Stem	15.3	18	Stem	15.1
4	Root	15.6	18	Root	15.5
5	Leaf	14.7	19	Leaf	16.1
5	Stem	15.7	19	Stem	16.6
5	Root	14.6	19	Root	16.5
6	Leaf	14.8	20	Leaf	15.8
6	Stem	14	20	Stem	15.2
6	Root	14.9	20	Root	16
7	Leaf	14.8	21	Leaf	16
7	Stem	14.7	21	Stem	14
7	Root	15.8	21	Root	15.3
8	Leaf	14.5	22	Leaf	14.7
8	Stem	15.7	22	Stem	15.7
8	Root	14.8	22	Root	14.3
9	Leaf	14.6	23	Leaf	15.8
9	Stem	14	23	Stem	14.7
9	Root	15.3	23	Root	16
10	Leaf	14.5	24	Leaf	14.5
10	Stem	15.8	24	Stem	14.9
10	Root	15.8	24	Root	16.1
11	Leaf	14.9	25	Leaf	14.2
11	Stem	14.3	25	Stem	14.4
11	Root	14.2	25	Root	16.4
12	Leaf	15.4	26	Leaf	15.2
12	Stem	14.6	26	Stem	14.4
12	Root	15.9	26	Root	14.6
13	Leaf	15	27	Leaf	15.2
13	Stem	14.3	27	Stem	14.8
13	Root	15	27	Root	14.2
14	Leaf	15.5	28	Leaf	15.3
14	Stem	14.4	28	Stem	14.1
14	Root	15.4	28	Root	16.7

NMR analysis.

Once the metabolites of each phase were obtained from the leaves, stems, and roots of each of the 28 chards. Samples were resuspended with 700µL of NMR buffer. The NMR buffers differed depending on whether the samples came from the aqueous or lipid phase. The NMR buffer of the aqueous phase consisted of a D_2O phosphate buffer (PBS 0.05 mM, pH 7.4, 99,5%). In addition, this buffer contains about 15 mg of 0.73 mM trisilylpropionic acid (TSP), which is a reference molecule for the subsequent analysis, and a little sodium acid (NaN3) to prevent the growth of microorganisms. And the NMR buffer of the lipid phase contained 16:8:1 ratio of $CDCl_3$, CD_3OD, and D_2O, respectively. In addition, the lipidic buffer also carries a reference molecule, in this case was 1,18mM tetramethylsilane (TMS). When all samples had 700L of NMR buffer, they were vortexed and transferred into 5mm NMR glass tubes, which were capped and introduced into the NMR 600MHz Bruker® equipment. For aqueous extracts, one-dimensional ^1H pulse experiments were carried out using the nuclear Overhauser effect spectroscopy (NOESY) presaturation sequence (RD–90◦–t1–90◦–tm–90◦ ACQ) to suppress the residual water peak, and the mixing time was set at 100 ms. Solvent presaturation with an irradiation power of 75 Hz was applied during recycling delay (RD = 5 s) and mixing time. The 90◦ pulse length was calibrated for each sample. The spectral width was 12 kHz (20 ppm), and a total of 256 transients were collected into 64 k data points for each 1H spectrum. In the case of lipophilic extracts, a 90◦ pulse with a presaturation sequence (zgpr) was used to suppress the residual water signal of methanol. An RD of 5.0 s with an acquisition time of 2.94 s was used. The 90◦ pulse length was calibrated for each sample. After 4 dummy scans, a total of 128 scans were collected into 64K data points with a spectral width of 18.6 ppm (18).

The exponential line broadening applied before Fourier transformation was 0.3 Hz. The frequency domain spectra were phased, baseline-corrected, and referenced to trimethylsilyl propinate or TMS signal (δ = 0 ppm) using TopSpin software (version 3.2, Bruker).

Results processing

The metabolites were identified by HMDB, Chenomx NMR suite 8.4 software (Chenomx Inc., Edmonton, AN, Canada) (Table 2) (18). These were integrated using AMIX 3.9.15 software package for quantification and statistical analysis (Figure 1) using the formula [metabolite] = ((Metabolite integration/no. of protons of the integrated signal)/(eretic integration/no. of protons of eretic) x [eretic] x (700µL/mg heavy (Table 1)) = mM. And all statistical analysis (PCAs, Heatmap and ANOVA) were performed with MetaboAnalyst software 5.0 (https://www.metaboanalyst.ca/).

C. elegans culture

Plates were prepared with an agar medium containing agar, NaCl, peptone, $MgSO_4$, $CaCl_2$, KH_2PO_4, K_2HPO_4, cholesterol, bi-distilled water. 500 µl of E. coli was added in agar medium plates. Once E. coli were dry, C. elegans n2 strain was used as our control strain (wild type) and C. elegans RB2510 as our problem strain (RB2510 strain was made by OMRF Knockout Group with EMS mutagen and the genotype W08D2.5(ok3473) IV. (https://cgc.umn.edu/strain/RB2510)). They were made in triplicate. After the worms were incubated for 3 days. Sample were synchronized when there were enough egg worms. For synchronization, M9 buffer was used which is obtained by diluting M9 x10 in 450mL of autoclaved bi-distilled water and 500µL of autoclaved $MgSO_4$. M9 x10 is composed of KH_2PO_4, Na_2HPO_4, NaCl and bi-distilled water. Considering that E. coli could interfere our results, three washes were done with M9 buffer. Worms and worm eggs were transferred to 15mL Falcons®. Each wash was performed using 15ml of M9 buffer and centrifugated at 1,500rpm for 1 min at 20°C. Supernatant was discarded since the pellet contained the worms and their eggs. Only 1ml of buffer was used to resuspend the pellet in the last wash. In order to not reduce the egg worm population, the synchronization must not take more than 10 minutes. And it was done by adding 2mL of a bleaching solution to the pellet. The bleaching solution contains 1mL of bleach, 2.5mL of 1M NaOH and 0,5mL of bi-distilled water. The Falcons® had to be shaken vigorously for 3 minutes and then fill until 15mL of M9 buffer. Following that, they were centrifuged at 1.500rpm for 1 minute at 20°C, discarding the supernatant. Finally, the Falcons® were washed again 3 times, like a mention above and in the last wash, we left approximately

2mL of M9 buffer with worm eggs. Finally, the Falcons® are left in rotation to allow the worms to oxygenate before performing the experiment (19).

Experimental part of C. elegans

For the experimental part, 8.000 worms were used. Synchronized C. elegans were lyophilized and made the polar extraction in Falcons®. After that, the aqueous phase was transferred to 2mL Eppendorfs®. The extraction methods of the metabolites were the same used at plants, although with some changed steps since, between vortexing and centrifugation, instead of waiting 15 minutes at room temperature, we waited 10 minutes at 4ºC. In addition, in the centrifugation the rpm was lower, in total 12.000rpm. In addition, the NMR buffer for both the aqueous and lipid phases were the same as in plants, but in the end the NMR equipment used was 500MHz instead of 600MHz.

C. elegans results processing

In this case, we were more interested in knowing the differences between one strain and another. So, first PCAs and OPLSs were performed using Matlab R2022a. The metabolites that in OPLSs were significant were identified using HMDB, Chenomx NMR suite 8.4 software (Chenomx Inc., Edmonton, AN, Canada). The PCAs and OPLSs were performed using the whole spectra, aligned, and normalized, with the regions of TMS, TSP, water, methanol and chloroform deleted (18).

RESULTS

After alignment and normalization of the spectra a total of 44 metabolite were found. In the aqueous phase, 33 metabolites were identified and integrated, and 11 metabolites in the lipidic phase (Table 2).

Table 2.- ^{1}H NMR resonance assignment with chemical shift and multiplicity of the metabolites found in chards. (F-Leaf; P-Stem; A-Root) (s-singlet; d-doublet; t-triplet; m-multiplicity; dd-double doublet; dt-double triplet).

Metabolite	Source	ppm	Coupling Type	Nº of protons
AQUOSE PHASE				
1,4-Butanediammonium	P	3.03-3.06	t	4
3-Methylxanthine	F, P, A	8.02-3.03	s	1
4-Aminobutyrate	F, P, A	2.28-2.32	t	2
Adenine	F, P, A	8.23-8.25	s	1
Alanine	F, P, A	1.47-1.49	d	3
Aspargine	P	2.86-2.89	d	2
Betaine	F, P, A	3.26-3.28	s	9
Choline	F, P, A	3.20-3.21	s	9
cis-Aconitate	A	5.67-5.72	s	1
Ethanolamine	F, P, A	3.13-3.16	t	2
Formate	F, P, A	8.45-8.46	s	1
Fructose	F, P, A	4.10-4.13	d	2
Fumarate	F, P, A	6.51-6.53	s	2
Gallate	F, P	7.05-7.07	s	2
Glucose	F, P, A	4.63-4.66 (β); 5.22-5.25 (α)	d; d	1; 1
Glutamate	F, P, A	2.02-2.09	m	1
Glutamine	F, P	2.43-2.48	m	2
Guanosine	F, P	7.99-8.00	s	1
Isoleucine	F, P	1.00-1.02	d	3
Lactate	F, P, A	1.32-1.34	d	3
Leucine	F, P	0.96-0.98	d	3
Malate	F, P, A	2.65-2.70	dd	3
Orotic Acid	F	6.13-6.23	s	1
Phenylalanine	F	7.36-7.43	dt	3
Succinate/Oxalacetat	F, P, A	2.40-2.41	s	4
Sucrose	F, A	5.40-5.43	d	1
Terminal -CH3 (Phytol)	A	0.62-1.23	m	3
Trigonelline	F	8.82-8.86	t	2
Tyrosine	F, P	7.18-7.21	d	2
UDPs	F	5.98-6.04	m	1
Uracil	F	7.53-7.55	d	1
Uridine	F, P, A	5.89-5.93	dd	2
Valine	F, P	1.03-1.06	d	3
LIPIDIC PHASE				
Monoglycerides	F	4.88-4.91	q	1
MUFAs	F, P, A	5.27-5.44	m	2
PUFAs	F, P, A	2.80-2.86	m	2
Total FA Chains	F, P, A			
% Saturated FA	F, P, A			
% Unsaturated FA	F, P, A			
% MUFA	P, A			
% PUFA	P, A			
% LINOLEIC	F, P, A	2.76-2.80	t	2
% OLEIC	F, P, A	2.00-2.03	m	4
% OTHERS LIPIDS	F, P, A			

The PCA methodology was used to confirm that there are differences between groups. The groups were leaf (F), stem (P), and root (A) of chards (Figure 1 a)). The logarithmic PCA was performed to see which metabolites were more characteristic of each part of the chard (Figure 1 b)). And the Heatmap was used to see in a different way than PCA the clusters between the parts of the chard and to see which parts were more similar to each other. The results showed that the root is more similar between leaf and stem than these two are to each other (Figure 1 c)). The PCAs and Heatmap were performed using the whole spectra, removing the TSP or TMS, methanol, chloroform, and water regions in both phases, aqueous and lipidic.

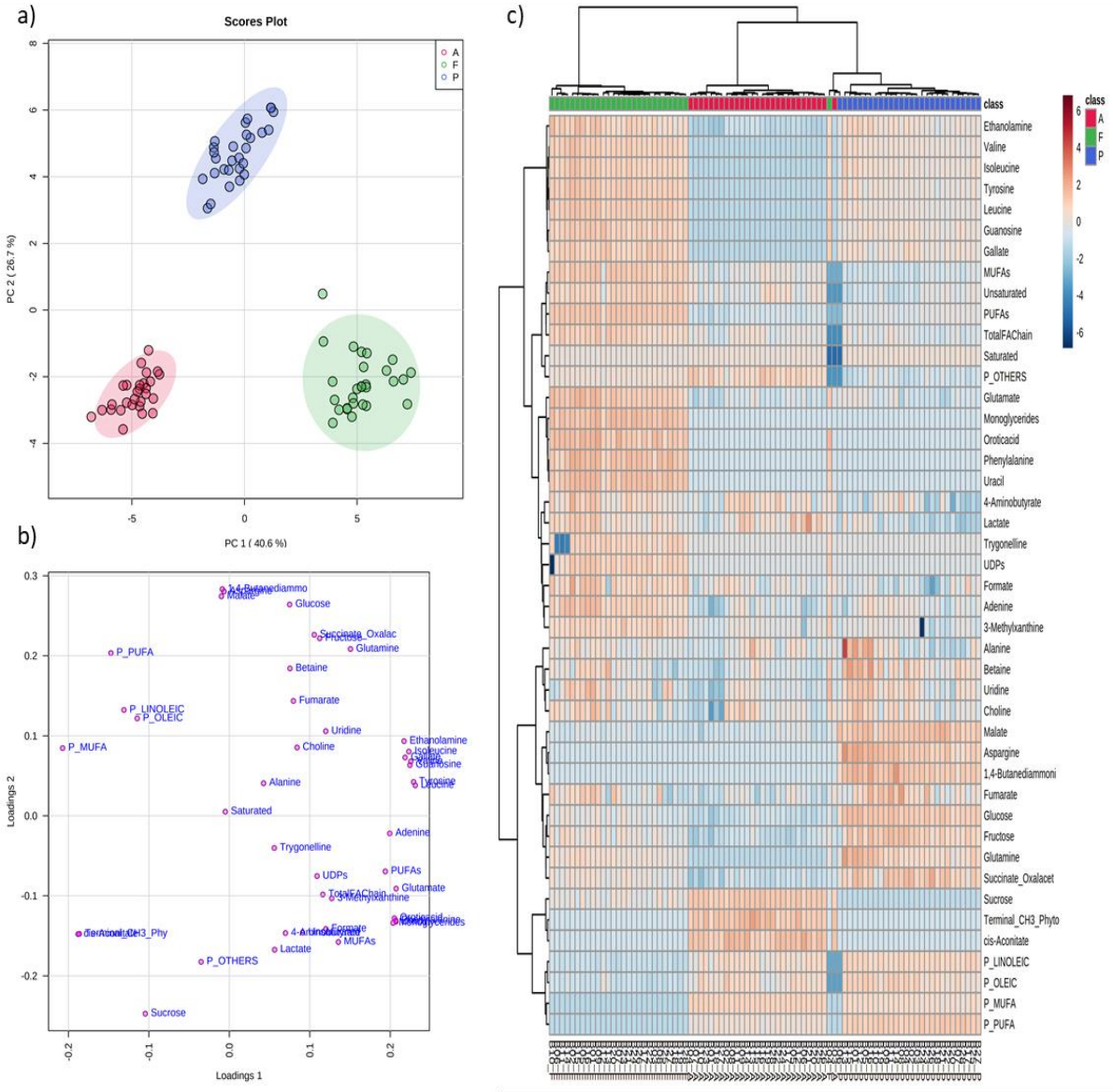

Figure 1.- a) PCA of chards (F-leaf (green); P-stem (blue); A-root (red)); b) PCA loading of chards; c) Heatmap of chards (F-leaf (green); P-stem (blue); A-root (red)).

After integrated and calculated the concentrations of each metabolite. An ANOVA test with Tukey's HSD were performed to find out which metabolites were significant ($p<0,05$). The results showed that 41 of 44 metabolites were significant and only 3 compounds were unsignificant. Also, the Tukey's HSD post-hoc test explains the significant relations of the metabolites between chard parts (Table 3).

Table 3.- ANOVA results of the comparison between all chard parts (F-leaf; P-stem; A-root). FDR < 0.05 were considered statistically significant.

Metabolites	f.value	p.value	-log10(p)	FDR	Post-hoc test Tukey's HSD
Leucine	1300.8	2.73E-62	61.563	1.20E-60	F-A-A-F
Guanosine	1115	1.15E-59	58.94	2.53E-58	F-A-A-F
cis-Aconitate	984.56	1.47E-57	56.833	1.85E-56	F-A-A
Uracil	981.15	1.68E-57	56.775	1.85E-56	F-A-F
Orotic Acid	968.86	2.74E-57	56.562	2.41E-56	F-A-F
Valine	964	3.33E-57	56.477	2.44E-56	F-A-A-F
Phenylalanine	955.57	4.69E-57	56.329	2.95E-56	F-A-F
Terminal -CH3 (Phytol)	909.02	3.26E-56	55.487	1.79E-55	F-A-A
Tyrosine	848.72	4.65E-55	54.333	2.27E-54	F-A-A-F
Sucrose	739.19	9.53E-53	52.021	4.09E-52	F-A-A-F
Gallate	737.82	1.02E-52	51.99	4.09E-52	F-A-A-F
Isoleucine	713.51	3.70E-52	51.432	1.36E-51	F-A-A-F
1,4-Butanediammonium	618.73	8.53E-50	49.069	2.89E-49	P-A-F
Monoglycerides	522.14	5.22E-47	46.283	1.64E-46	F-A-F
Aspargine	395.17	1.64E-42	41.784	4.82E-42	P-A-F
% MUFA	345.14	2.30E-40	39.638	6.32E-40	F-A-A-F
% PUFA	344.35	2.50E-40	39.602	6.46E-40	F-A-A-F
Glucose	315.84	5.64E-39	38.249	1.38E-38	F-A-A-F
Malate	296.83	5.19E-38	37.284	1.20E-37	P-A-F
Glutamine	231.87	3.01E-34	33.522	6.62E-34	F-A-A-F
Ethanolamine	224.44	9.22E-34	33.035	1.93E-33	F-A-A-F
Glutamate	186.58	4.75E-31	30.323	9.51E-31	F-A-F
Succinate/Oxalacetat	120.87	4.84E-25	24.315	9.27E-25	F-A-A-F
Fructose	110.3	7.52E-24	23.124	1.38E-23	F-A-A-F
PUFAs	85.781	9.95E-21	20.002	1.75E-20	F-A-A-F
Adenine	69.092	3.10E-18	17.509	5.24E-18	F-A-A-F
% LINOLEIC	49.6	8.62E-15	14.064	1.41E-14	F-A-F
MUFAs	45.48	5.74E-14	13.241	9.02E-14	F-A-A-F
Formate	31.906	6.04E-11	10.219	9.17E-11	F-A-A-F
% OLEIC	31.159	9.19E-11	10.037	1.35E-10	F-A-F
Lactate	29.891	1.89E-10	9.7225	2.69E-10	P-A-F
3-Methylxanthine	24.224	5.67E-09	8.2462	7.80E-09	F-A-F
% OTHERS	23.963	6.68E-09	8.175	8.91E-09	P-A-F
4-Aminobutyrate	22.118	2.17E-08	7.6645	2.80E-08	P-A-F
% Unsaturated FA	18.977	1.74E-07	6.7592	2.19E-07	F-A-F
Betaine	18.921	1.81E-07	6.7425	2.21E-07	F-A-A-F
Total FA Chain	15.768	1.65E-06	5.7837	1.96E-06	F-A-F
Fumarate	15.173	2.53E-06	5.5967	2.93E-06	F-A-A-F
UDPs	14.58	3.91E-06	5.4083	4.41E-06	F-A-F
Uridine	11.684	3.48E-05	4.4585	3.83E-05	F-A-A
Choline	3.3709	3.92E-02	1.4062	4.21E-02	
Trygonelline		Unsignificant			
% Saturated FA		Unsignificant			
Alanine		Unsignificant			

With C. elegans we did a PCA and OPLS's. After alignment, and normalization, PCA were performed to see if there are differences between control strain metabolites and problem strain (Figure 2). The result of the PCA shows that in component 1 are differences between N2 strain and RB strain. And the OPLSs models show what peaks are significant for the control strain (below the spectra) and problem strain with PD knockout (above the spectra). The significance of these peaks is indicated by the blue colour on them. Those metabolites altered in the aqueous phase are: branched-chain amino acids (BCAA), lactate, alanine, glutamate, choline, phosphocholine, betaine, glycine, glycerol, allantoine, ATP/ADP/AMP, tyrosine, and phenylalanine (Figure 3 a)). In lipidic phase, only αH2 fatty acids (FA), βH2 FA and triglycerides presented changes (Figure 3 b)).

The PCA and OPLS were performed using the whole spectra removing the TSP or TMS, methanol, chloroform, and water regions in both phases, aqueous and lipidic.

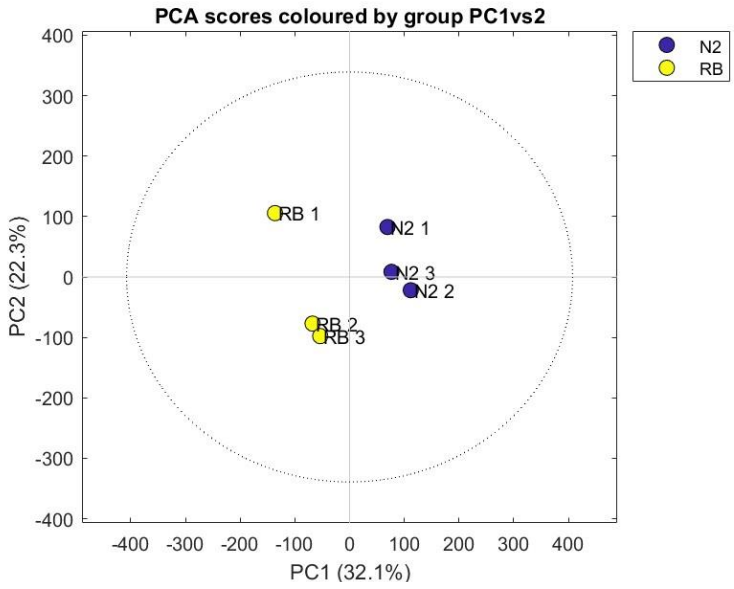

Figure 2.- PCA of C. elegans (N2-Control strain; RB-Parkinson Strain)

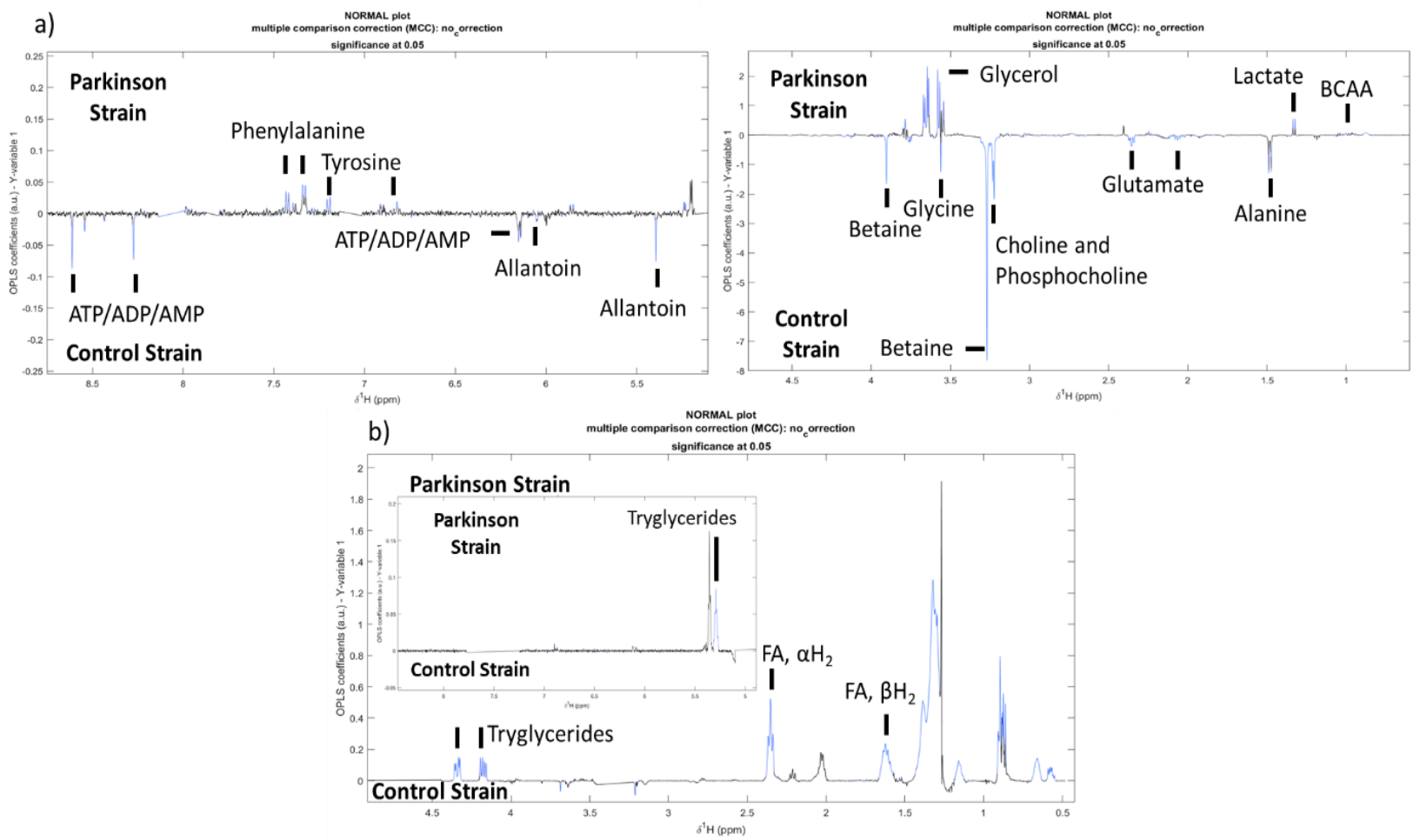

Figure 3.- OPLS of C. elegans with metabolites identification (a) Aqueous phase; b) Lipidic phase). The superior parts of the spectras represent the PD strain (RB2510) and the inferior ones represent the control strain (n2). Blue peaks indicates if the peak is significant (p-value<0.05).

DISCUSSION

In the present study, we showed that there are differences between leaves, stems, and roots of the chards (Figure 1 a)) and that differences are also in their metabolic system since almost all metabolites were significant. The great number of significant metabolites that differ between the three chard regions remarked how different are their metabolism (Table 3). The most important metabolites in the multivariant PCA model in leaves are orotic acid, phenylalanine, uracil and monoglycerides; in stems are 1,4-butanediamine or also called putrescine, asparagine, and malic acid; and, finally, in roots are cis-aconitate and the region of -CH3 terminals of the NMR spectra. About the last one, we did not know what compound or compounds could be, but we think that could be phytol. These new compounds discovered could have beneficial effects for humans.

It has been shown that the differentiating compounds of chard leaves may have an antibacterial effect. This antibacterial effect may be given by L-phenylalanine, uracil conjugates and monoglycerides (20–22). L-phenylalanine has been observed to be a good antimicrobial against gram positive bacteria. It is also associated with the retardation of the effects of lipopolysaccharides (LPS) (20). Uracil derivatives have been shown to be antibacterial, as well as antiviral, antiparasitic and anticancer (21). In case of monoglycerides, they have been observed to have good antibacterial potential against bacterial skin infections (22). We mentioned above that uracil derivatives may have anticancer effects and related to cancer. In this sense, orotic acid is also related with the synthesis of pyrimidines in the liver, and it has been evaluated as a possible ligand against liver cancer (23).

The effects of the differential compounds found in the stems differ from those found in the leaves.

A moderate amount of putrescine has been observed to increase, in general, the growth rate (24). The consumption of high concentrations of putrescine is toxic, but the levels of putrescine have been tested so as not to endanger the organism. This has also been observed in plants. And in plants, putrescine has been demonstrated to prolong the

storage capacity and increase the shelf life of plums (25). Additionally, in mice it has been shown to have an antidepressant effect (26).

In a study of asparagine supplementation, it increased muscle capacity to utilize free fatty acids (FFA) instead of glycogen, which was useful for prolonged exercise and reduced exhaustion (27).

And finally, L-malic acid was shown to prevent myocardial ischemia/reperfusion injury in rats, thus acting as an anti-inflammatory and antioxidant in certain signalling pathways (28). In addition, it was observed that in rats, malic acid also has a neuroprotective effect (29).

As we mentioned at the beginning of the discussion. The NMR region of the terminal -CH3 has not been identified to any specific compound or compounds. But we have determined that it could be phytol, although it will be confirmed by 2D and MS experiments. Also, this assumption of phytol is not very precise either because in that region there are many peaks that may correspond to different metabolites as well as to different structures of phytol, since this region belong to terminal -CH3 which are present in many different structures. Phytol is a part of the chlorophyll molecule considered to be the most abundant acyclic isoprenoid in the world. Phytol has many uses in cosmetics (used in fragrances), pharmaceuticals and biotechnologies (30). In addition, phytol and its derivatives have been shown to have cytotoxic anxiolytic, metabolism modulating, antioxidant, autophagy, and apoptosis inducing, antinocive, immunomodulatory, antimicrobial and, anti-inflammatory effects (31). This anti-inflammatory effect could also come from cis-aconitate since it, through itaconitate, influences immunity as an anti-inflammatory (32).

These effects have been described in the bibliography, but in order to test these effects and discover new ones, it would be appropriate to administer chard to an animal model, such as C. elegans.

There are different models of C. elegans with which to perform different experiments in metabolomics, but we have focused on models with PD since it was seen that in chard there are betalains (33). These betalains are involved in the dopamine pathway, an important molecule in PD.

The NMR metabolomics study of the of C. elegans with PD shown that are differences between the control strain and the problem control (Figure 3). And If we look a bit closer to see what triggers these differences, in the OPLSs we see those certain metabolites, either because they are found in higher or lower concentrations, differ between strains (Figure 4). Those metabolites as we mentioned before are in aqueous phase, BCAA, lactate, alanine, glutamate, choline, phosphocholine, betaine, glycine, glycerol, allantoine, ATP/ADP/AMP, tyrosine, and phenylalanine (Figure 4 a)). In the other side, those significant metabolites that appear in lipidic phase are αH2 FA, βH2 FA and triglycerides (Figure 4 b)).

These metabolites differences between strains show a difference in the metabolome or a metabolic change between both the physiological and the pathological state. It has been already demonstrated that altered BCAA levels are related to the metabolism of PD, since a metabolic disruption of them would cause motor deficits and neurodegeneration, both present in PD (34). Also, it has been studied that glutamate receptors are involved in many actions of neurons, therefore, a deregulation of the glutamate concentration might cause an alteration of neurotransmission in PD (35). In addition, it is known that PD presents oxidative stress, which with adequate levels of betaine could protect the cerebellum from the oxidative stress of PD (36).

Altogether, suggest that this C. elegans strain RB2510 is suitable for future PD-related metabolomics studies using NMR.

CONCLUSIONS

Responding to the objectives of this project, we can conclude that we have identified new metabolites in the different chards and that these metabolites may have a beneficial effect on health. In a first step, to see new effects, they must be tested on animal models, like C. elegans, as this has once again proven to be a very suitable and economically inexpensive model. Eventually and, depending on the results of these studies, they would be tested on humans. In addition, C. elegans has been shown to have very good differential results, metabolically speaking, to be used as a model for PD in metabolomics studies, since the wild type and the PD strain present different metabolomic signatures.

Although we have made these statements, further metabolomic studies need to be performed to find out new beneficial effects of the compounds found in the chard. Moreover, other technics need to be used, as MS or centrifugal partition chromatography, to confirm the realised assignment of those metabolites that do not appear in any data base.

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