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Preliminary Investigation on Salivary Profiling of Filipino Women Using High Performance Liquid Chromatography

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Abstract: Saliva is a complex and dynamic biological fluid which performs many different functions in the oral cavity. But as new technology arises, many more compounds can be identified in saliva such as pollutants, hormones, drugs and biomarkers. Thus, saliva was used as a potential media for metabolomics study in cancer research. In this study, high performance liquid chromatography (HPLC) was used to analyse the saliva samples from several Filipino women. A baseline metabolomic profile from 24 healthy individuals was created and 42 peaks were separated from the salivary samples. The concentrations, retention times and peak areas of the 42 separated peaks were also established. Furthermore, the optimization of salivary sample preparation and analysis were done for the use of High performance Liquid Chromatography.

Key Words: salivary profiling; metabolomics; biomarkers; high performance liquid chromatography

1. INTRODUCTION

Metabolomics involves the analysis of a biofluid, in this case saliva, to determine specific metabolites that will aid physicians to diagnose whether or not a person has a certain disease, such as breast cancer.

This study focuses on optimizing a method for analyzing and profiling the different metabolites present in the saliva of healthy female Filipino subjects. This study also aims to determine the baseline of present metabolites in healthy individuals residing in the Philippines, specifically, people in the Metro Manila area. Comparison with this baseline will later serve as a reference data when studying the metabolites of breast cancer patients. Note that all other forms of cancer are not within the scope of this study.

The study involves the collection and analysis of saliva; as an alternative to the other invasive procedures for determining whether an individual has breast cancer. Also as opposed to other methods of biofluid collection, saliva collection is cost-effective, easy to collect, and painless. The study also involves an instrumental analysis of the saliva samples. The analyses will be done using the High Performance Liquid Chromatography (HPLC) system and to determine the relative amounts of

the metabolite present, the use of an internal standard will be utilized.

The results of the study will be valuable in the early identification of breast cancer for pre-emptive purposes. The baseline, if proven and retested, will help in the determination of the severity of the cancer or the determination of whether an individual is inflicted with the carcinoma or not. Moreover, the metabolomic study could provide a cheaper and a non-invasive diagnosis and monitoring of certain diseases such as cancer.

2. METHODOLOGY

2.1 Saliva Sampling

The population of possible volunteers was limited to healthy Filipino females who are in the age range of 35 to 50 years. The population was further limited to non-smoking, non- medicating individuals with no diagnosed illness for the past months.

Prior to the saliva collection, plastic drinking straws were cut into 2-inch (5cm) pieces. The participants were also asked to rinse their mouth rigorously with water 10 minutes before the sample collection. Thereafter, the participants were given one piece of plastic straw, used as an aid in the saliva collection, and one cryovial, labeled with their respective sample number.

Due to the possibility of foaming or bubbling of the saliva, participants were instructed to allow saliva to pool in their mouth to minimize the foaming. Then participants were asked to tilt their heads forward so as to allow the saliva to flow down the straw and pool in the 5-ml centrifuge tube (passive drool) (salimetrics, 2012). This was repeated until 5-mL sample is collected. Approximately, 10 – 15 minutes was the duration time for the total sample collection.

2.2 Sample Preparation

After collecting 5-mL of salivary samples, each sample was centrifuged at 1,500 rpm for 60 mins. at 4^{o}C. Thereafter, the supernatant was collected and was transferred to a 25-mL cryovial. The samples were hydrolyzed with 1:1 volumes of 0.1 M hydrochloric acid. Then after the addition of the acid, the sample mixture was ultrasonicated for 30 mins. The samples were then freezed and lyophilized to dryness overnight. When dried, saliva samples were reconstituted with Mobile Phase A, 0.1% formic acid in water. The samples were then filtered and were then transferred to HPLC injection vials.

2.3 Analysis Using High Performance Liquid Chromatography

After sample preparation, the following were the chromatographic separation parameters used. A reversed phase column, Restek Pinnacle II C18 (250 x 4.6 mm, 5um particle size), was utilized for the separation of the components of the salivary sample. A sample injection volume of 10uL and a flow rate of 1.000mL/min were also utilized. Mobile phases: 0.1% formic acid in (A) acetonitrile and (B) water were used in the analysis

Table 2-1: ***Summary of the Chromatographic Gradient***

CHROMATOGRAPHIC GRADIENT		
Time (mins)	**% Mobile Phase A** 0.1% Formic Acid in Water	**% Mobile Phase B** 0.1% Formic in Acid in Acetonitrile
5.00	98.0	2.0
40.00	30.0	70.0
55.00	10.0	90.0
70.00	0.0	100
80.00	0.0	100
90.00	98.0	2.0
Stop Time: 90 minutes		Temperature: 27^{o}C

2.4 Analysis Using an Internal Standard

A single internal standard was utilized in order to identify the concentrations of the different metabolites

present in the sample. In order to do this, a stock solution of 10,000 ppm phenol was made and was used to create a 1,000 ppm phenol solution in the sample injection vials. A total of 500uL-solution of sample and the internal standard, having a 1:9 volume ratio was prepared and was injected for the analysis using High Performance Liquid Chromatography. The addition of the internal standards was only performed during the determination of the concentrations from the actual samples collected.

2.5 Data Processing

The data collected were processed using the ChemStation software by Agilent Technologies. All of the retention times and their respective peak areas were recorded, summarized and compared. However, significant peaks were identified. These peaks may be used for future reference and studies. These significant peaks are characterized by their frequency of occurrence, the intensity of the peak areas, and the intensity of the peak height.

The volunteer for all the tests the determination of the optimum conditions in the analysis of salivary sample is a healthy individual. This was done in order to reflect the metabolic profile of the wild-type Filipino women and to at least have a consistent metabolic profile.

3. RESULTS AND DISCUSSION

According to study done by Alvarez, some hormones, lipids, amino acids, and several metabolic intermediates, have been found in analyzed saliva samples; and the resulting metabolites depend on the method of preparation and analysis of the sample. In the present study, using High Performance Liquid Chromatography, the metabolites were not conclusively identified. However, a baseline metabolite profile and corresponding concentrations were created instead. An internal standard was used to determine the average concentration of each peak detected by the HPLC.

Phenol was used as the internal standard due to its polarity and molecular weight. In terms of its polarity, it is not too polar (American Laboratory, 2012) and is eluted in the middle of the chromatogram. Its molecular weight is also close to the target analytes, which are mostly amino acids. In addition, phenol is not reactive with the components of the saliva samples. Furthermore, the 1000 ppm phenol standard appears at about a retention time of 19 minutes. This does not overlap with any peaks of interest from previous analyses. Amino acids and other vitamins are not considered as internal standards for they may be a biomarker for breast cancer even though their relative structures and properties are almost the same as the analytes.

Table 3-6: *The Metabolomic Profile*

Peak	Average Retention Time	Minimum Peak Area	Maximum Peak Area	Median Peak Area	Average Peak Area	Average Concentration (ppm)	Percent Frequency
1	2.48138	22.39682	1673.88074	428.15035	574.27824	111.13322	100.00
2	2.61267	3.83957	418.85788	54.20343	158.96696	30.52162	12.50
3	2.67383	5.16448	46.85970	16.55817	20.30158	3.96721	25.00
4	2.75550	8.59871	19.34122	13.96997	13.96997	2.96274	8.33
5	2.81033	3.42410	10.38917	6.04251	6.61859	1.28992	12.50
6	2.95975	5.44547	112.53492	8.44154	22.52592	4.59460	33.33
7	3.32014	8.30601	112.85026	23.52128	31.85067	5.92311	58.33

8	3.14271	7.99733	36.22366	15.46318	17.29426	3.46571	29.17
9	3.34892	454.89981	4099.05957	1746.62293	1928.12478	377.65057	100.00
10	3.49600	266.96545	266.96545	266.96545	266.96545	46.12931	4.17
11	3.64230	6.52174	262.60608	116.67489	118.59347	22.48135	41.67
12	3.91517	47.95968	264.18948	95.73240	117.52522	22.98584	25.00
13	4.42967	28.14877	52.80076	32.92065	37.95673	7.58908	12.50
14	4.78175	28.36121	159.96584	72.43569	83.29961	17.73718	16.67
15	5.49400	23.90906	23.90906	23.90906	23.90906	5.06184	4.17
16	5.95750	32.70399	96.70935	64.70667	64.70667	13.03742	8.33
17	6.59750	8.85982	40.05634	27.91989	26.18898	4.73594	16.67
18	7.04800	7.78964	7.78964	7.78964	7.78964	1.58425	4.17
19	7.57750	28.26493	151.65181	89.95837	89.95837	17.67739	8.33
20	9.34333	12.53747	45.14849	28.50971	28.73189	5.10856	12.50
21	10.00875	11.45733	1359.17371	140.36512	412.84032	78.66045	16.67
22	10.27900	7.34790	72.97495	41.99914	41.08661	7.88588	25.00
23	11.23320	9.05080	26.79925	19.93109	17.96500	3.54557	20.83
24	11.59500	17.73708	17.73708	17.73708	17.73708	3.63167	4.17
25	12.09700	7.85728	7.85728	7.85728	7.85728	1.60878	4.17
26	12.73468	6.38721	240.80263	36.04683	64.04674	12.17026	91.67
27	13.06100	6.32464	40.78416	10.55489	19.22123	3.81036	12.50
28	14.09520	8.74020	122.08025	37.28544	47.99899	9.38933	83.33
29	14.32100	7.78828	11.17408	9.48118	9.48118	1.92486	8.33
30	15.10620	1.10858	20.72143	7.64281	10.15561	1.74345	20.83
31	15.86000	5.20632	68.68423	17.54298	22.43199	4.14210	41.67
32	16.23209	9.01518	468.74142	74.89813	120.43834	23.39368	95.83
33	17.08978	6.30250	38.81585	11.98427	15.25034	2.89150	37.50
34	17.55167	1.77195	7.42597	6.06803	5.08865	0.94828	12.50
35	18.58171	8.04156	40.38031	18.17876	20.01142	3.63705	29.17
36	19.37350	4517.6231	8010.00098	5005.52100	5175.03078	1000	100.00
37	21.86950	37.77490	38.42657	38.10074	38.10074	7.42622	8.33
38	24.04833	2.88145	19.39963	12.97875	11.75328	2.40435	12.50
39	26.75600	4.06575	14.30723	9.18649	9.18649	1.81684	8.33
40	43.25639	32.04565	244.03601	98.51810	148.35163	29.33325	95.83
41	52.77800	99.12294	99.12294	99.12294	99.12294	17.86038	4.17
42	56.89947	412.94662	1123.99951	875.32001	762.83476	150.99122	79.17
43	59.54100	18.61246	18.61246	18.61246	18.61246	3.95451	4.17

* the blue rows represent the significant peaks and the orange row represents phenol

The characterization of the salivary metabolite peaks: the peaks were assigned with arbitrary numbers according to their retention times, peak areas, and/or peak height. Once grouped together, their average retention time, minimum, maximum, median peak area, average peak area, average concentration and frequency of occurrence were determined; these values can be found in Table 3-6.

As can be seen from the data in Table 3-6, most of the peaks have a low frequency of occurrence and low concentration. However, there are about 8 peaks that are consistently appearing in every sample, excluding the internal standard. Among these 8, there are 4 that have relatively high peak areas these are peak numbers 1, 9, 21 and 42. These peaks not only have high peak areas, they also have high percent frequency of occurrences. These peaks may be used as specific metabolites for the analysis for future studies.

Peaks 10, 15, 18, 24, 25, 41 and 43 have low percent frequency of occurrence. These peaks are considered to be products of degradation that may be bacterial in nature. Possible sources of degradation in the samples include the introduction of microorganisms which could degrade the sample, the difference in the environment, the delay in the preparation and drying of the samples, and the post-drying storage.

The average concentrations of the metabolites are shown in Table 3-6. Upon the determination of the concentrations of the metabolites in the saliva samples, it was found that the saliva samples have greater concentrations relative to the levels of the concentrations of metabolites found in a different study (Al-Shehri, Henman, Charles, Cowley, Shaw, Liley, Tomarchio, Punyadeera & Duley, 2013). This difference in the results may be attributed to the time of collection of the salivary samples. As stated in the previous discussion, the biological rhythm would be able to alter the concentrations of the metabolites in the sample. Unlike the study done by Al-Shehri and team, this study utilized a pre-concentration step in order to increase the metabolite concentrations and to exhaust all possible metabolites that could possibly be a biomarker for breast cancer.

The data collected from the analyses show the metabolite profile of healthy Filipino women. These data comprise the baseline values for the concentrations of the metabolites. The maximum, minimum and the average values from the data could still change if a greater population is tested and their salivary samples analyzed. Furthermore, the results from the present study could be used only as a guide for future studies regarding the salivary metabolomic profile of healthy Filipino women. However, variation from the values obtained from this study could not conclude any diagnosis for any diseases. To do this, an even more intensive clinical study must be done.

Individual identities of the metabolites have not been differentiated in the present study. But succeeding studies could be able to use the preliminary data presented as means of approximating the retention times, the separation, and the concentration of the metabolites.

4. CONCLUSIONS

This study aims to profile the different salivary metabolites of healthy Filipino women. The study was able to successfully optimize the conditions for the sample preparation, storage and handling. The optimum HPLC conditions for the separation of salivary metabolites was also established.

The salivary metabolite profile of healthy subjects was successfully compared. Approximate values of the minimum, maximum, median, average values and frequency of occurrence were determined based on internal standard. The range of concentrations of the metabolites commonly occurring among healthy Filipino female subjects were established.

5. REFERENCES

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