Supporting Information

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Antioxidant, anticholinesterase, and tyrosinase enzyme inhibitory profiles of nine Saudi honeys revealed by multivariate analysis

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Determination of hydroxymethylfurfural

Hydroxymethylfurfural (HMF) is determined in a clear, filtered, aqueous honey solution using reverse phase HPLC equipped with UV detection. The signal is compared with those from standards of known concentration. This method described by (Jeuring and Kuppers[15]).

1. Reagents

Mobile phase: water-methanol (90+10 by volume), both HPLC quality. Standard solutions: 5-(hydroxymethyl-) furan-2-carbaldehyde (HMF), (e.g. Merck No. 820 678 or Fluka No. 55690), 1, 2, 5 and 10 mg /L aqueous solution. The solution should be prepared on the day of use. Determination of standard HMF-content The absorbance A of the prepared standard solution is determined using an UV spectrophotometer at 285 nm in 1 cm quartz cells with water in the blank cell. The concentration of the standard solutions can be calculated from the literature values for molar absorptivity, ε = 16830 or absorptivity, a1cm 1% = 133.57 (3). Concentration in mg/L = A x x 1 13357 1 000. , where A is the absorbance of the standard solution. The calculated content must correspond to the specifications given by the supplier. The standard has to be stored at 4 - 8 °C under nitrogen. It is extremely hygroscopic.

2. Equipment

Liquid chromatograph with UV detector and integrator. Column: any column with C18-reversed phase material. e.g Hypersil ODS 5 µm, 125 x 4 mm or 250 x 4 mm. Membrane filter, 0.45 µm (e.g. Dynagard).

3. Procedure

Preparation of samples

Accurately weigh about 10 g of prepared honey sample into a 50 ml beaker. Dissolve the sample in approx. 25 ml of water and transfer quantitatively to a 50 ml volumetric flask. Dilute to 50 ml with water. Filter through a 0.45 µm membrane filter to provide a sample solution ready for chromatography. Conditions for chromatography flow rate 1.0 ml/ minute quantity injected 20 µL of sample or standard solution. Detection UV 285 nm; range: 0.2 AUFS.

4. Calculation and Expression of Results.

The HMF content of the sample is calculated by comparing the corresponding peak areas of the sample and those of the standard solutions, taking into account the dilution. There is a linear relationship between the concentration and the area of the HMF peak. Results are expressed in mg/kg, to 1 decimal place.

Determination of sugars by HPLC

1. Reagents

Chemicals of analytical purity grade should be used. The water must be distilled or should be of at least equivalent purity. Methanol for HPLC Acetonitrile for HPLC Warning: Acetonitrile is a dangerous substance. Laboratory safety guidelines on dangerous substances at work should be consulted. Eluent solution for the HPLC. Mix 80

volumes of acetonitrile with 20 volumes of water. Degass prior to use. The standard substances, fructose, glucose and sucrose were purchased from sigma supplies. Pipette 25ml methanol into a 100 ml calibrated flask. Depending on the sugars to be analysed, dissolve the amounts detailed below in approximately 40ml water and transfer quantitatively to the flask and fill to the mark with water. Fructose: 2.000 g glucose: 1.500 g sucrose g Use a syringe and a pre-mounted membrane filter to transfer the solution to sample vials. The standard solutions are stable for 4 weeks in the refrigerator at 40 C and for six months at -180 C.

2. Equipment

Sample vials. Ultrasonic bath. Calibrated flasks, volume 100 ml, 25-ml-pipette. Membrane filter for aqueous solutions, pore size 0.45 µm. Filter holder for membrane filters with suitable syringe. High Performance Liquid Chromatograph consisting of pump, sample applicator, temperature regulated RI-detector thermostated at 30o C*, temperature regulated column oven at 300 C, integrator. Analytical stainless-steel column, e.g. 4.6 mm in diameter, 250 mm length, containing aminemodified silica gel with 5-7 µm particle size. Before use, carry out a system suitability test to ensure all the sugars can be separated.

3. Procedure

Preparation of samples

Preparation of the sample solution Weigh 5g of honey into a beaker and dissolve in 40 ml water. Pipette 25ml of methanol into a 100ml volumetric flask and transfer the honey solution quantitatively to the flask. Fill to the mark with water. Pour through a membrane filter and collect in sample vials. Store as for the standard solution. Flow rate: 1.3 ml/min mobile phase: Acetonitrile:water (80:20, v/v) column and detector temperature : 300 C sample volume: 10 µl Note: If it is not possible to carry out the analysis at 300 C and if the detector cannot be thermostated at 30o C, carry out the analysis at ambient temperature. In this case it is not possible to separate melezitose and erlose. Note: Identical volumes of sample and standard solution should be injected.

4. Calculation and Expression of Results

The honey sugars are identified and quantified by comparison of the retention times and the peak area of the honey sugars with those of the standard sugars. The mass percentage of the sugars, W, to be determined in g/100g is calculated according to the following formula (external standard procedure): W = A1 x V1 x m1 x 100/ A2 x V2 x m0 Where A1 = Peak areas or peak heights of the given sugar compound in the sample solution, expressed as units of area, length or integration. A2 = Peak heights of the given sugar compound in the standard solution, expressed as units of area, length or integration. V1 = Total volume of the sample solution in ml V2 = Total volume of the standard solution in ml m1 = Mass amount of the sugar in grams in the total volume of the standard (V2) m0 = sample weight in g.

Assays for Total Phenolic and Flavonoid Contents

The total phenolic content was determined by employing the methods given in the literature with some modification. Sample solution (0.25 mL) was mixed with diluted Folin–Ciocalteu reagent (1 mL, 1:9, v/v) and shaken vigorously. After 3 min, Na_2CO_3 solution (0.75 mL, 1%) was added and the sample absorbance was read at 760 nm after a 2 h incubation at room temperature. The total phenolic content was expressed as milligrams of gallic acid equivalents (mg GAE/g extract)(Uysal et al., 2017).

The total flavonoid content was determined using the $AlCl_3$ method. Briefly, sample solution (1 mL) was mixed with the same volume of aluminum trichloride (2%) in methanol. Similarly, a blank was prepared by adding sample solution (1 mL) to methanol (1 mL) without $AlCl_3$. The sample and blank absorbances were read at 415 nm after a 10 min incubation at room temperature. The absorbance of the blank was subtracted from that of the sample. Rutin was used as a reference standard and the total flavonoid content was expressed as milligrams of rutin equivalents (mg RE/g extract) (Uysal et al., 2017).

Determination of Antioxidant and Enzyme Inhibitory Effects

Antioxidant (DPPH and ABTS radical scavenging, reducing power (CUPRAC and FRAP), phosphomolybdenum and metal chelating (ferrozine method)) and enzyme inhibitory activities (cholinesterase (Elmann's method), tyrosinase (dopachrome method), α-amylase (iodine/potassium iodide method), α -glucosidase (chromogenic PNPG method) and pancreatic lipase (*p*-nitrophenyl butyrate (p-NPB)

method) were determined using the methods previously described by Uysal et al. (Uysal et al., 2017) and Grochowski et al. (Grochowski et al., 2017)

For the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay: Sample solution was added to 4 mL of a 0.004% methanol solution of DPPH. The sample absorbance was read at 517 nm after a 30 min incubation at room temperature in the dark. DPPH radical scavenging activity was expressed as milligrams of trolox equivalents (mg TE/g extract).

For ABTS (2,2′-azino-bis(3-ethylbenzothiazoline) 6-sulfonic acid) radical scavenging assay: Briefly, ABTS+ was produced directly by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and allowing the mixture to stand for 12–16 in the dark at room temperature. Prior to beginning the assay, ABTS solution was diluted with methanol to an absorbance of 0.700 ± 0.02 at 734 nm. Sample solution was added to ABTS solution (2 mL) and mixed. The sample absorbance was read at 734 nm after a 30 min incubation at room temperature. The ABTS radical scavenging activity was expressed as milligrams of trolox equivalents (mg TE/g extract).

For CUPRAC (cupric ion reducing activity) activity assay: Sample solution was added to premixed reaction mixture containing $CuCl_2$ (1 mL, 10 mM), neocuproine (1 mL, 7.5 mM) and NH_4Ac buffer (1 mL, 1 M, pH 7.0). Similarly, a blank was prepared by adding sample solution (0.5 mL) to premixed reaction mixture (3 mL) without $CuCl_2$. Then, the sample and blank absorbances were read at 450 nm after a 30 min incubation at room temperature. The absorbance of the blank was subtracted from that of the sample. CUPRAC activity was expressed as milligrams of trolox equivalents (mg TE/g extract).

For FRAP (ferric reducing antioxidant power) activity assay: Sample solution was added to premixed FRAP reagent (2 mL) containing acetate buffer (0.3 M, pH 3.6), 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ) (10 mM) in 40 mM HCl and ferric chloride (20 mM) in a ratio of 10:1:1 (v/v/v). Then, the sample absorbance was read at 593 nm after a 30 min incubation at room temperature. FRAP activity was expressed as milligrams of trolox equivalents (mg TE/g extract).

For phosphomolybdenum method: Sample solution was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The sample absorbance was

read at 695 nm after a 90 min incubation at 95 °C. The total antioxidant capacity was expressed as millimoles of trolox equivalents (mmol TE/g extract).

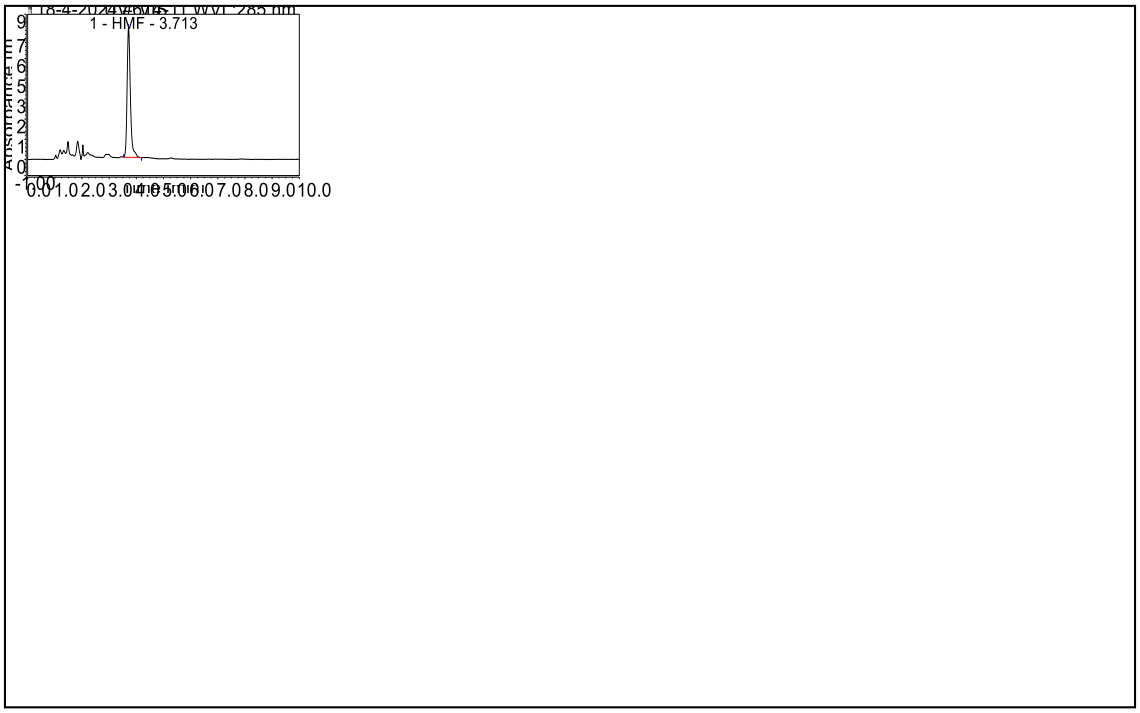
For metal chelating activity assay: Briefly, sample solution was added to $FeCl_2$ solution (0.05 mL, 2 mM). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). Similarly, a blank was prepared by adding sample solution (2 mL) to $FeCl_2$ solution (0.05 mL, 2 mM) and water (0.2 mL) without ferrozine. Then, the sample and blank absorbances were read at 562 nm after 10 min incubation at room temperature. The absorbance of the blank was sub-tracted from that of the sample. The metal chelating activity was expressed as milligrams of EDTA (disodium edetate) equivalents (mg EDTAE/g extract).

For Cholinesterase (ChE) inhibitory activity assay: Sample solution (was mixed with DTNB (5,5-dithio-bis(2-nitrobenzoic) acid, Sigma, St. Louis, MO, USA) (125 µL) and AChE (acetylcholines-terase (Electric ell acetylcholinesterase, Type-VI-S, EC 3.1.1.7,Sigma)), or BChE (butyrylcholinesterase (horse serum butyrylcholinesterase, EC 3.1.1.8, Sigma)) solution (25 µL) in Tris–HCl buffer (pH 8.0) in a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of acetylthiocholine iodide (ATCI, Sigma) or butyrylthiocholine chloride (BTCl, Sigma) (25 µL). Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme (AChE or BChE) solution. The sample and blank absorbances were read at 405 nm after 10 min incubation at 25 °C. The absorbance of the blank was subtracted from that of the sample and the cholinesterase inhibitory activity was expressed as galanthamine equivalents (mgGALAE/g extract).

For Tyrosinase inhibitory activity assay: Sample solution was mixed with tyrosinase solution (40 µL, Sigma) and phosphate buffer (100 µL, pH 6.8) in a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of L-DOPA (40 µL, Sigma). Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme (tyrosinase) solution. The sample and blank absorbances were read at 492 nm after a 10 min incubation at 25 °C. The absorbance of the blank was subtracted from that of the sample and the tyrosinase inhibitory activity was expressed as kojic acid equivalents (mgKAE/g extract).

High performance liquid chromatography (HPLC)) chromatograms of hydroxymethylfurfural

1.



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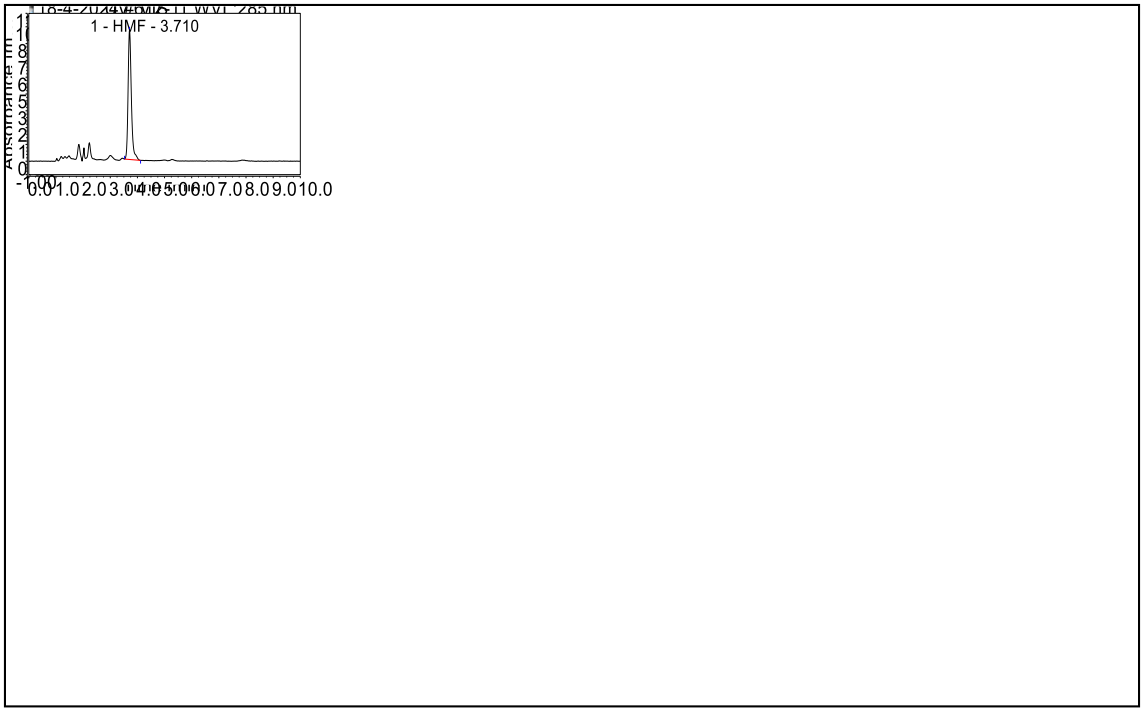

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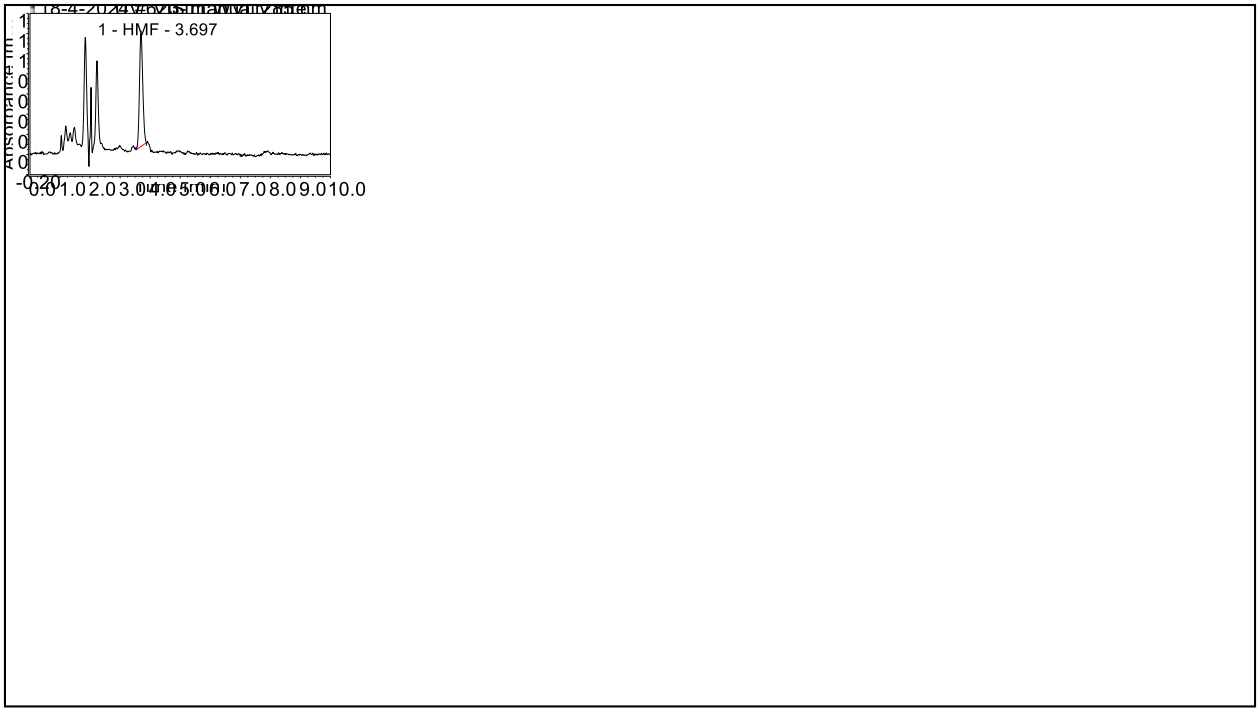

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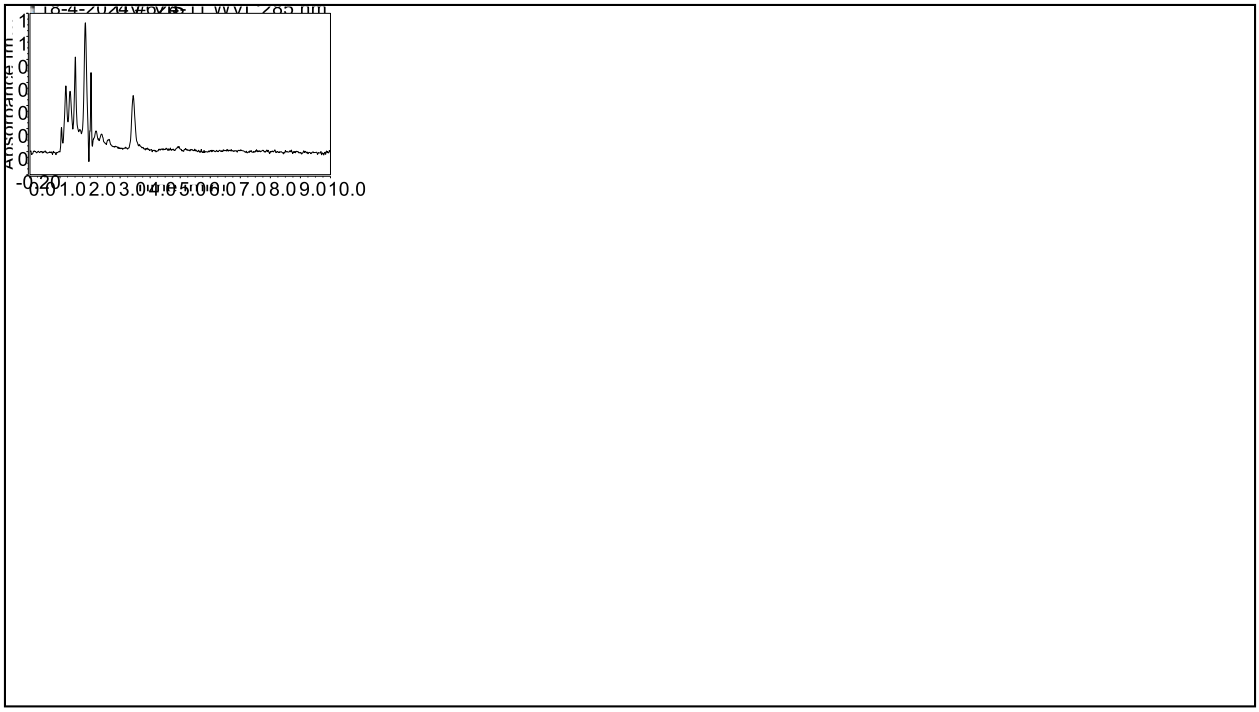

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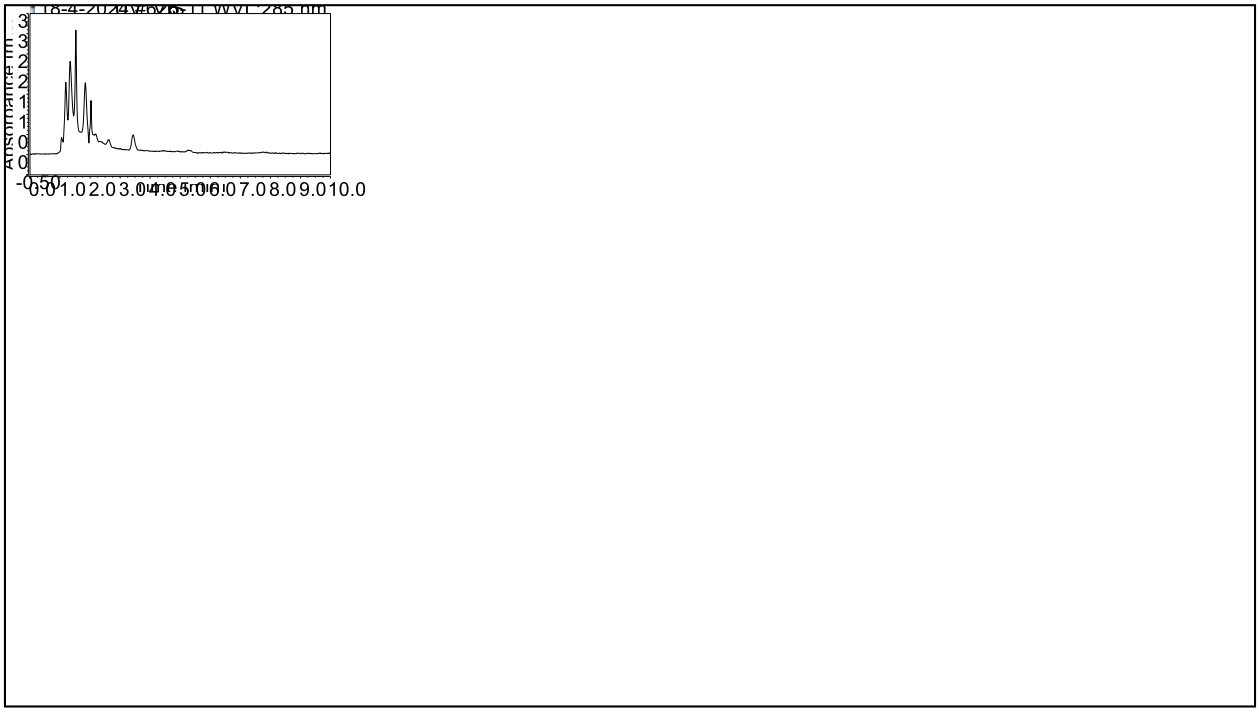

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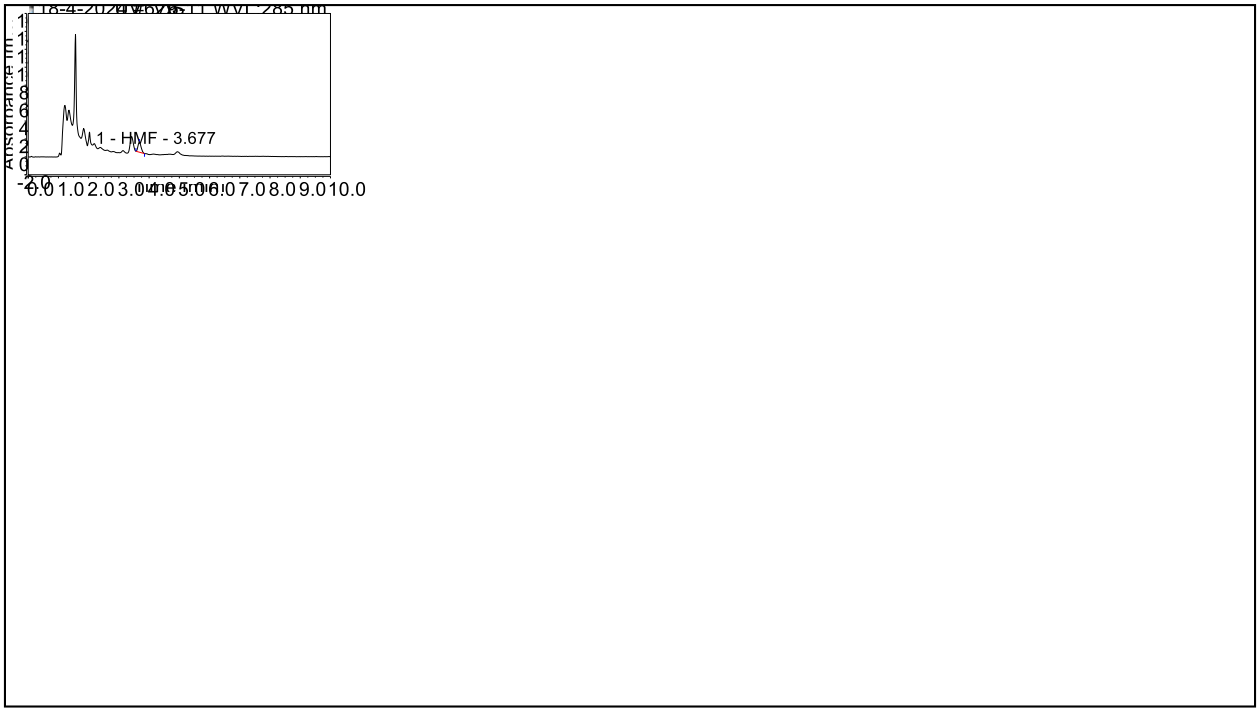

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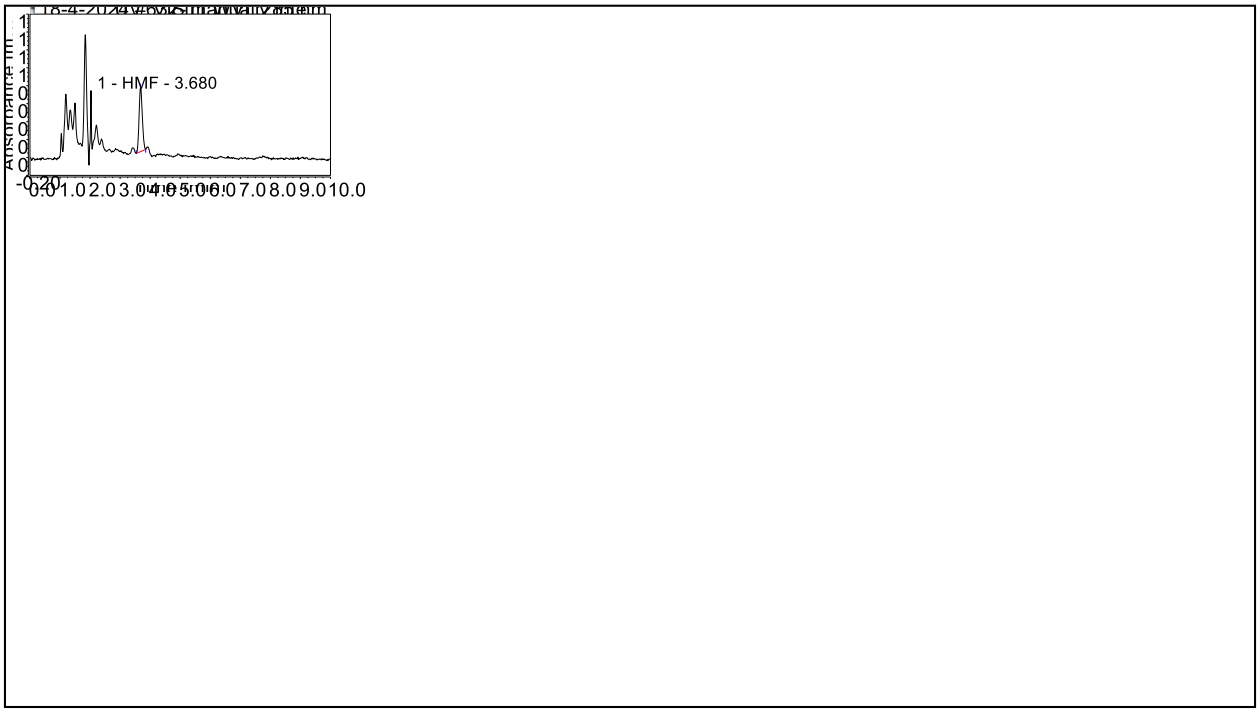

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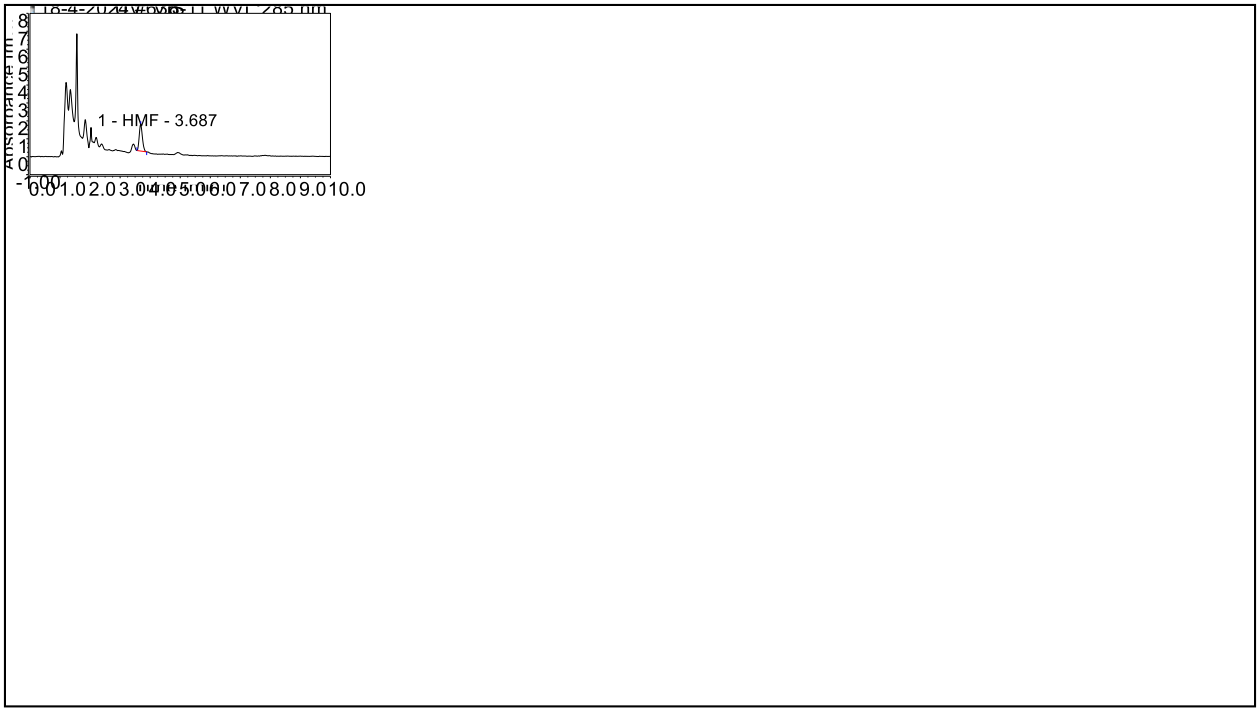

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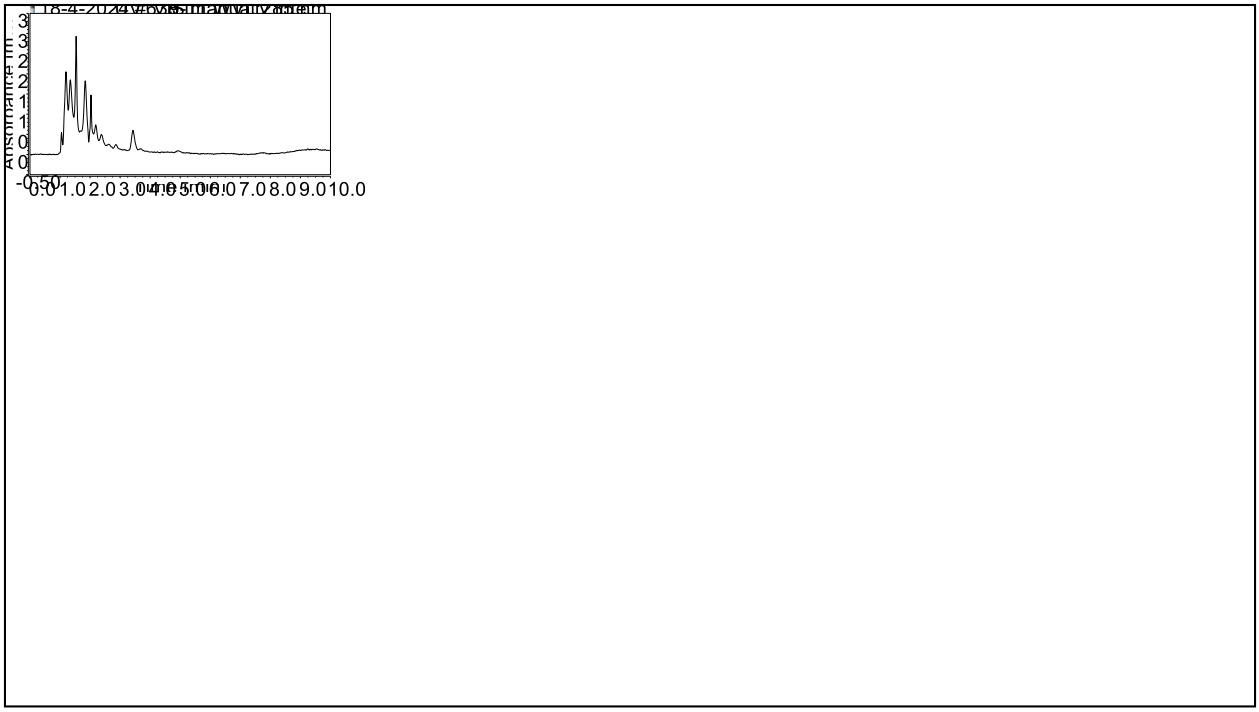
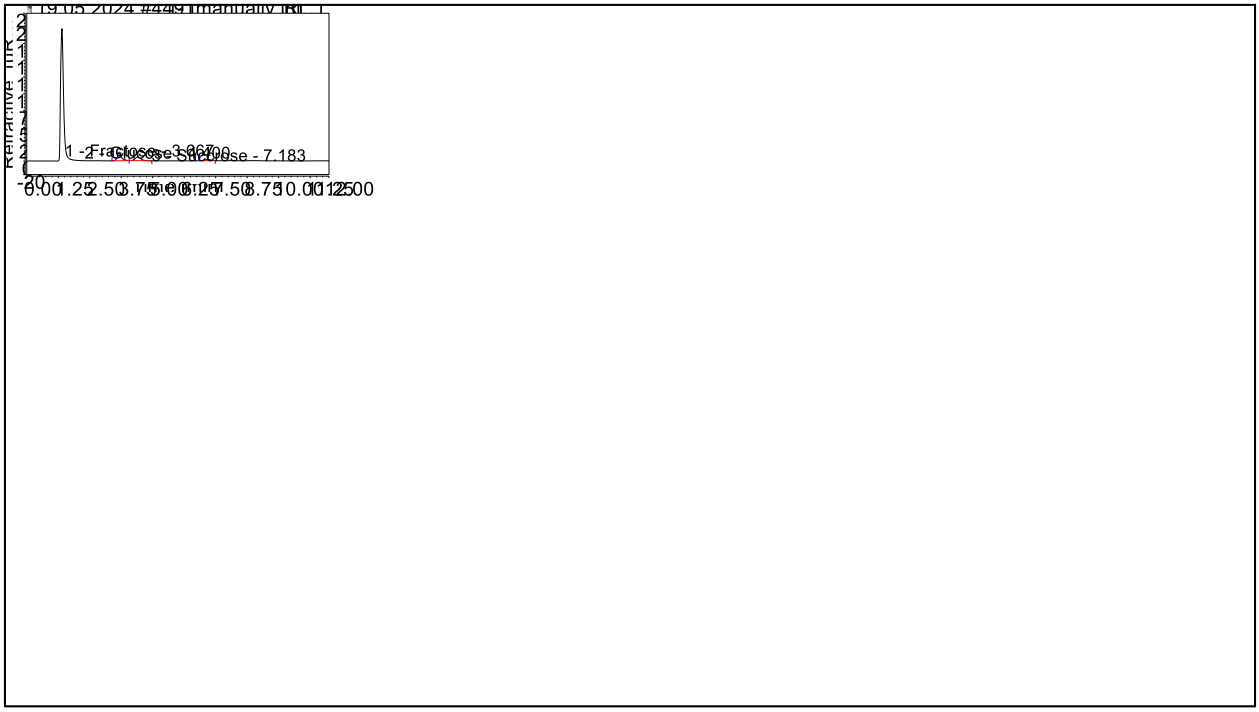


Figures S1: 1-9 showed (High performance liquid chromatography (HPLC)) chromatogram of hydroxymethylfurfural (HMF) from nine honey sample: 1; Talh Tehamh, 2; Qatada, 3; Majra, 4; Sider Tihamh, 5; Sider Albaha, 6;Talh Hail, 7; Sumra Bisha, 8; Dhuhyana, 9; Sider Bisha.

High performance liquid chromatography (HPLC)) chromatogram of fructose, glucose and sucrose

(1)



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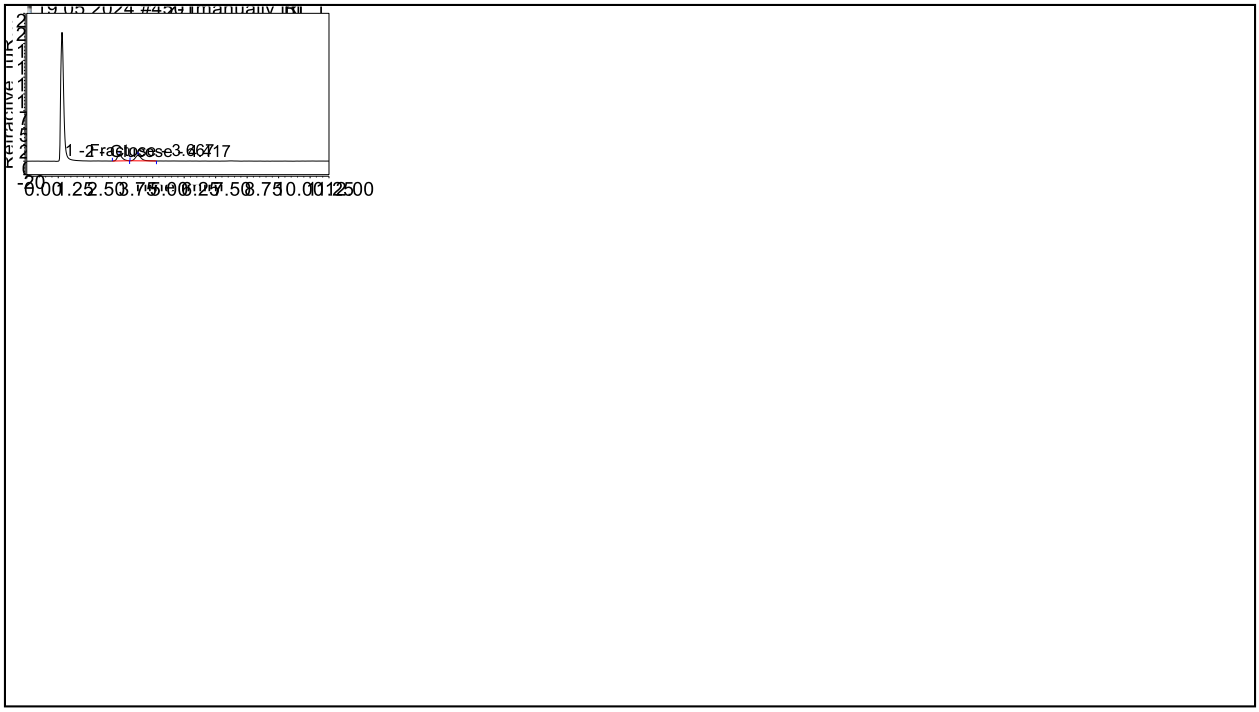

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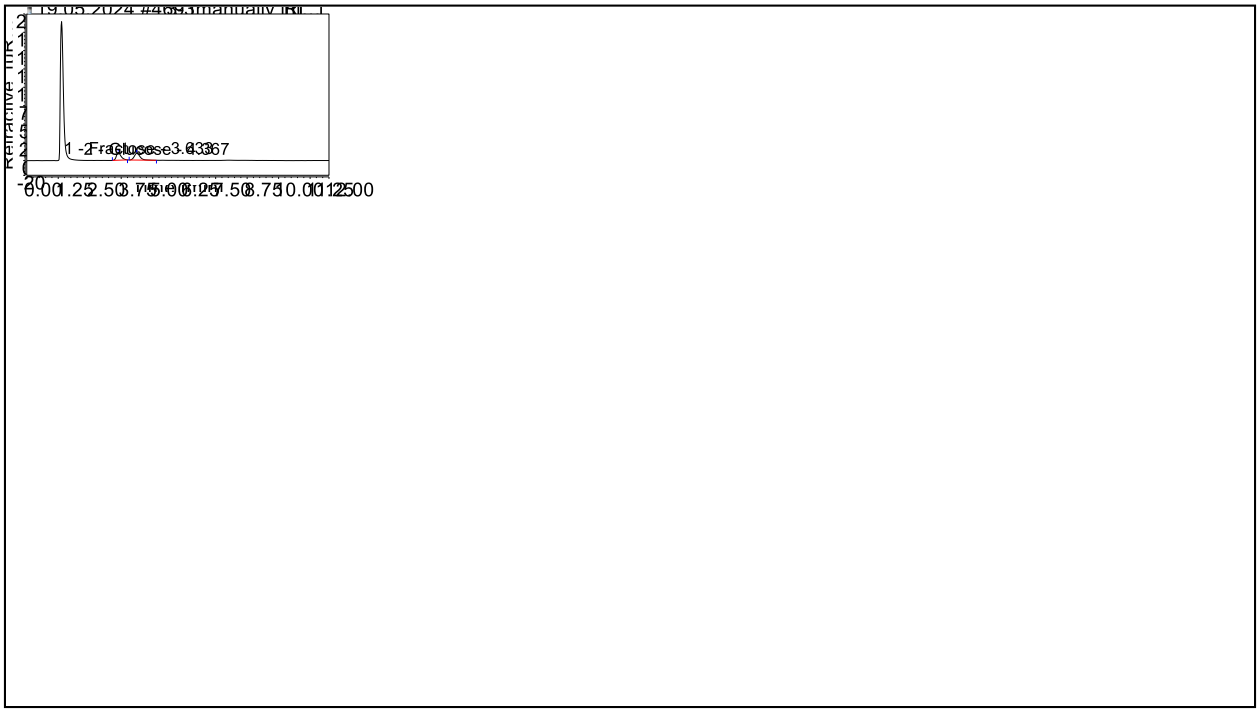

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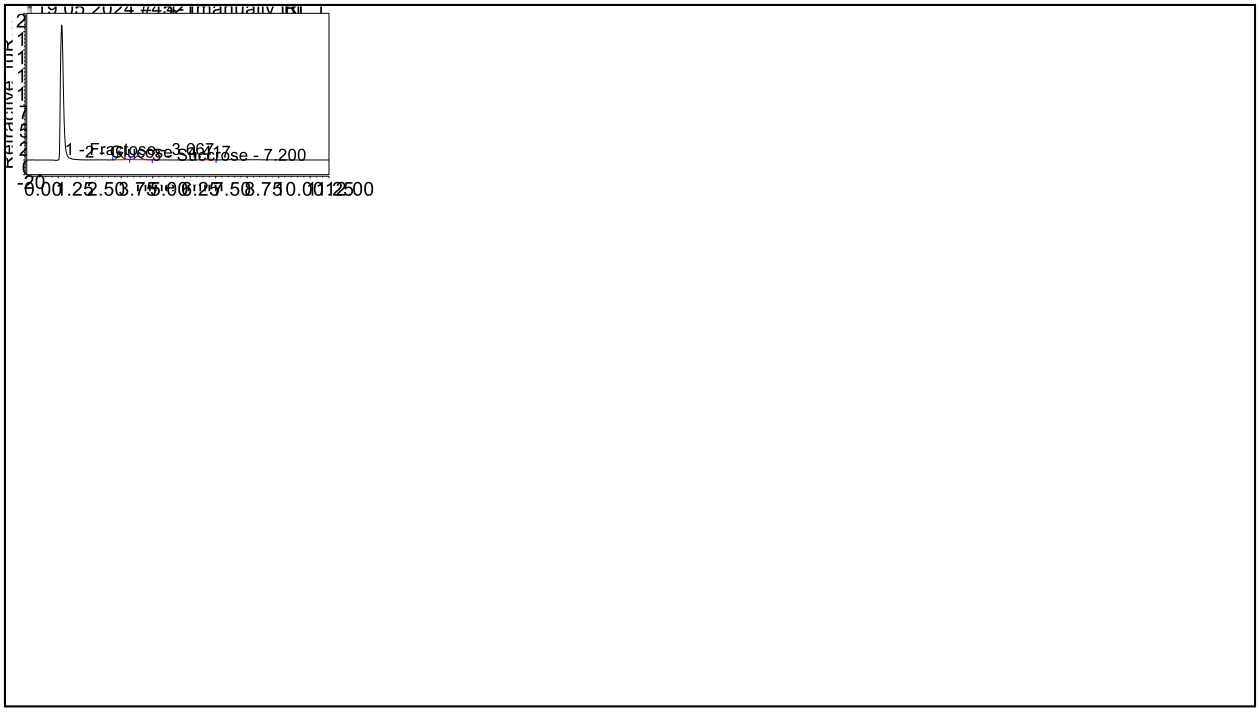

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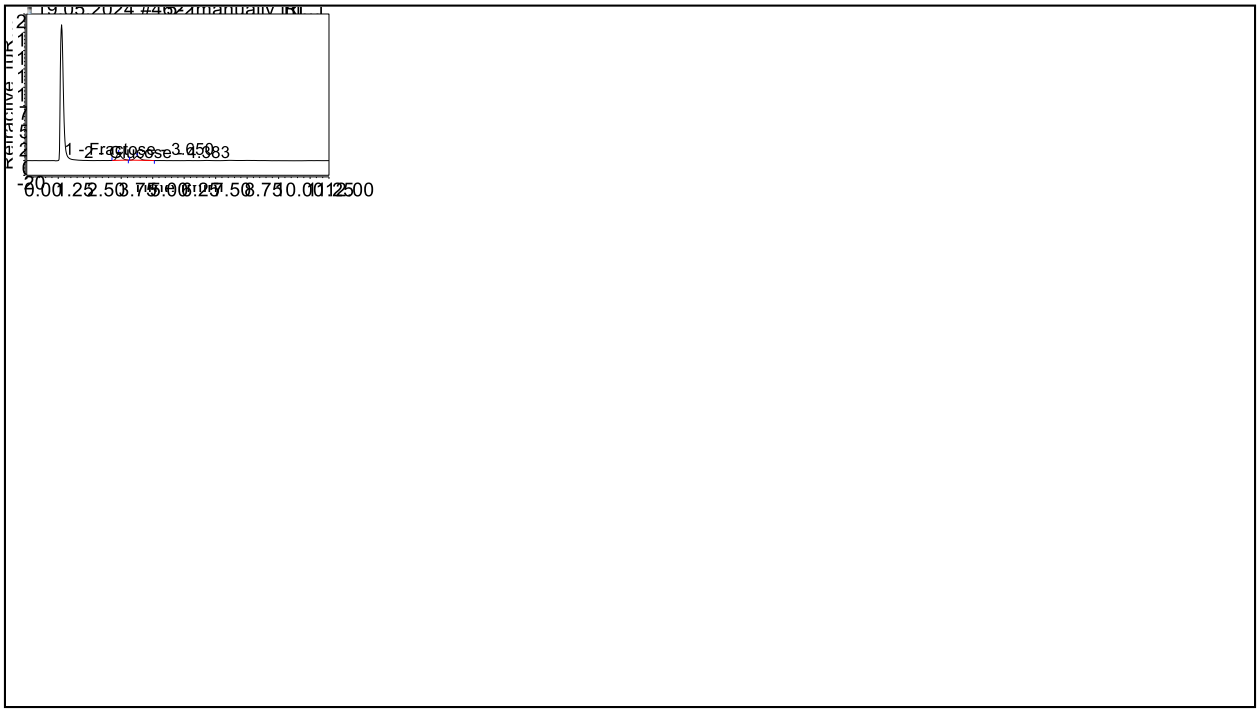

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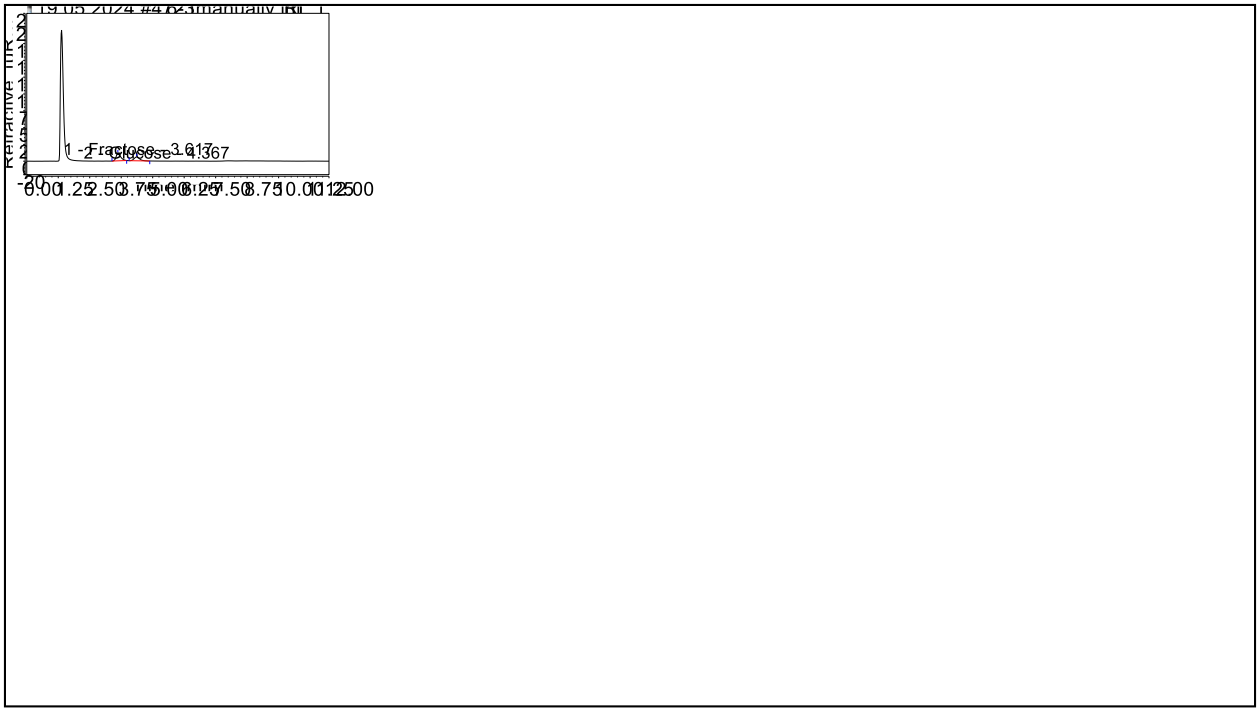

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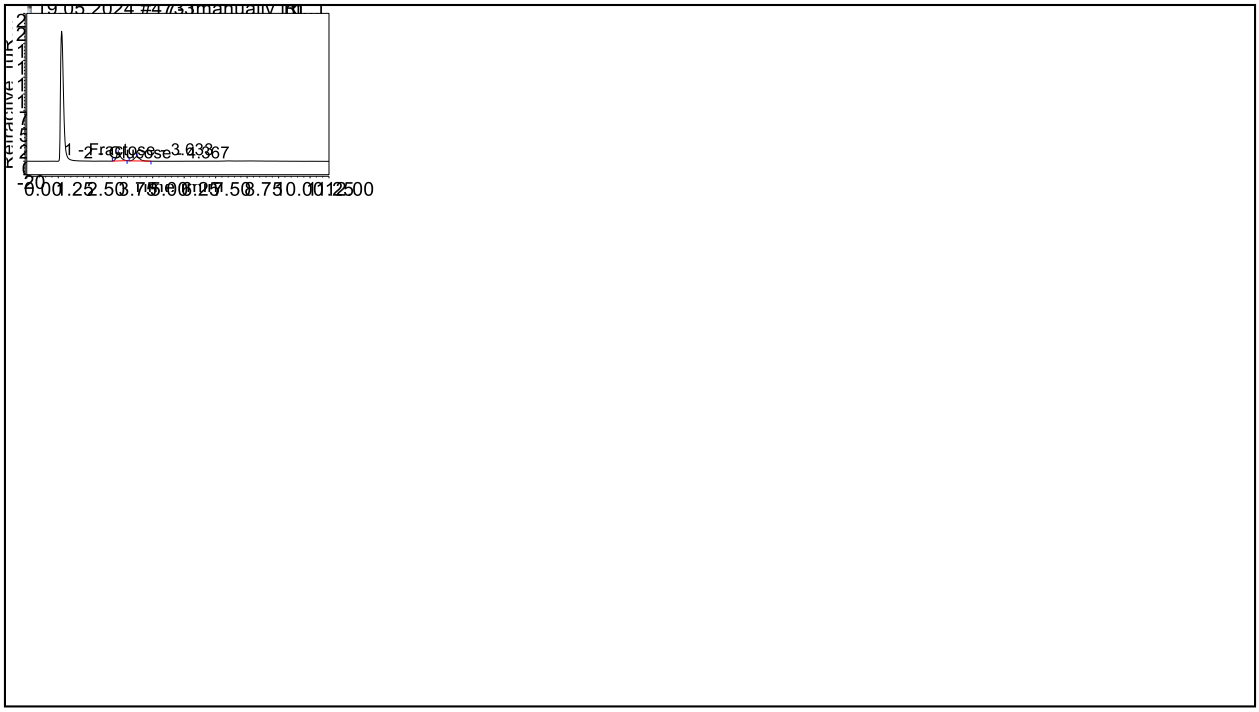

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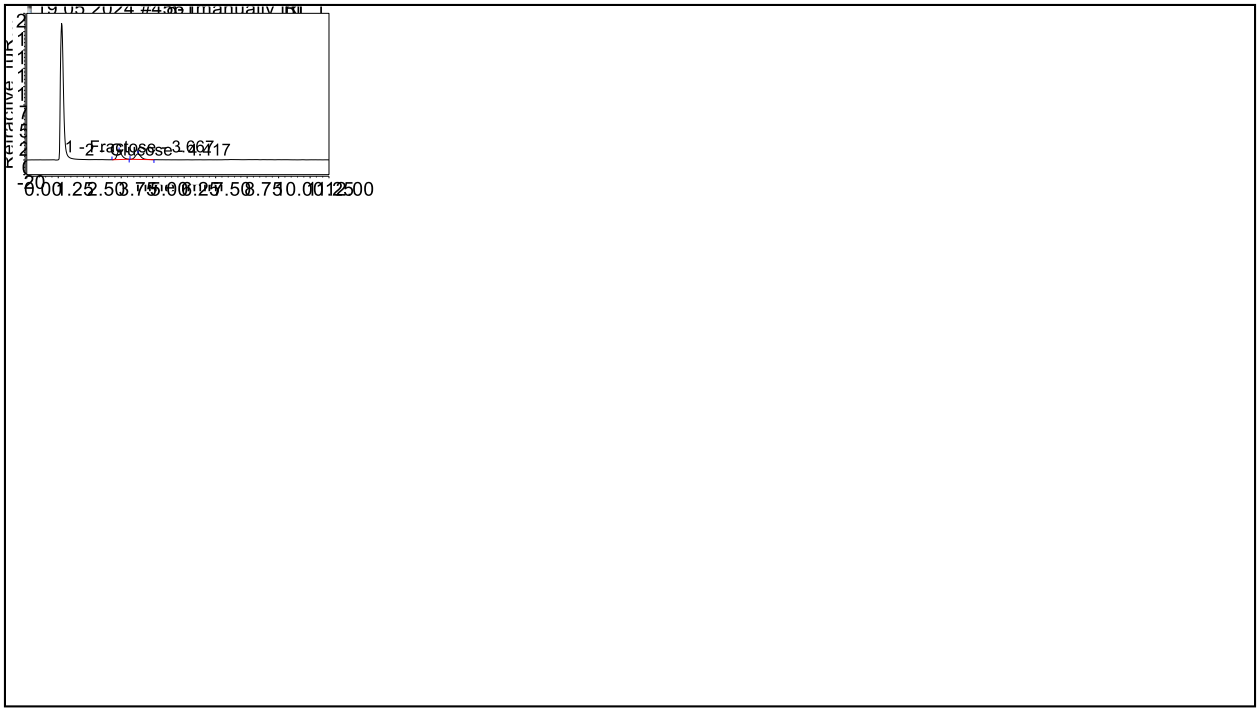

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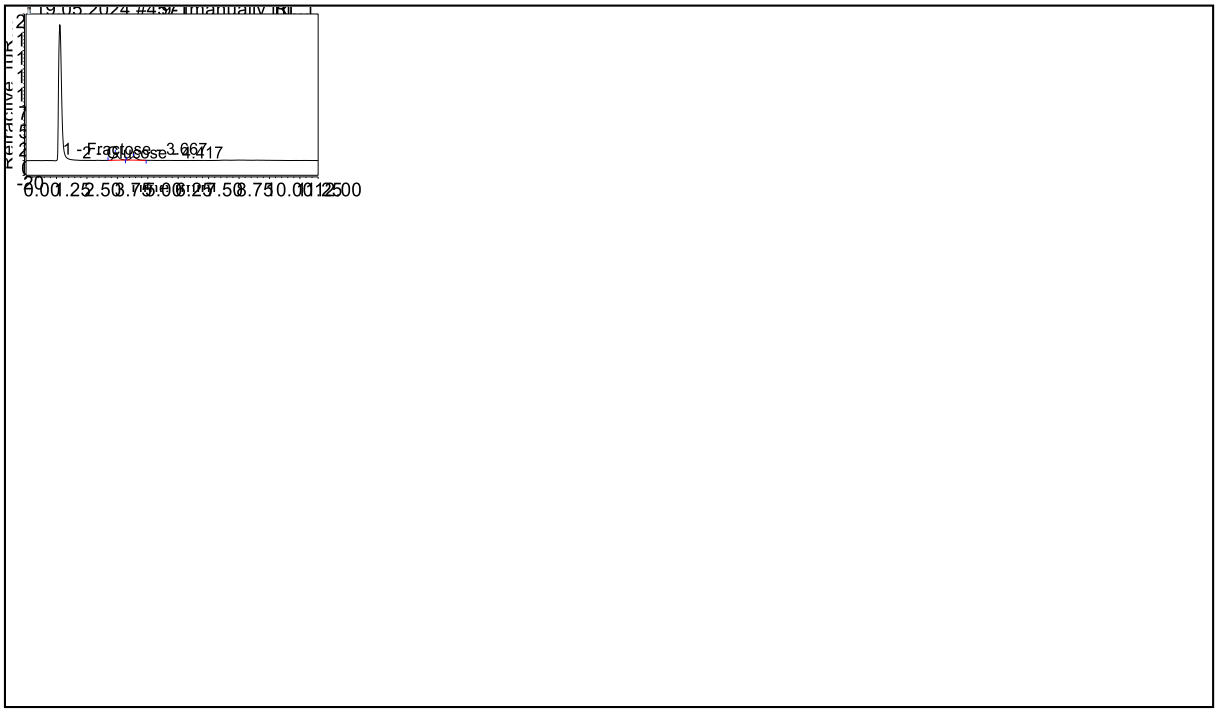

Figures S2: 1-9 showed (High performance liquid chromatography (HPLC)) chromatogram of fructose, glucose and sucrose from nine honey sample: 1; Talh Tehamh, 2; Qatada, 3; Majra, 4; Sider Tihamh, 5; Sider Albaha, 6; Talh Hail, 7;Sumra Bisha, 8; Dhuhyana, 9;Sider Bisha.

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