Vibrational Spectroscopy 84 (2016) 38–43

Contents lists available at ScienceDirect

Vibrational Spectroscopy

journal homepage: www.elsevier.com/locate/vibspec

Analytical method development using FTIR-ATR and FT-Raman spectroscopy to assay fructose, sucrose, glucose and dihydroxyacetone, in *Leptospermum scoparium* nectar

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ARTICLE INFO

Article history:
Received 7 September 2015
Received in revised form 22 February 2016
Accepted 27 February 2016
Available online 3 March 2016

Keywords:
Dihydroxyacetone
Leptospermum scoparium
FT-Raman
FTIR-ATR
Nectar composition

ABSTRACT

The carbohydrate dihydroxyacteone (DHA) occurs in significant levels in *Leptospermum scoparium* (mānuka) nectar and is the precursor of methylglyoxyl (MGO), the unique non-peroxide antibacterial activity (NPA) component in mānuka honey. The nectar of ten different cultivars of *L. scoparium* was assayed quantitatively for fructose, glucose, sucrose, and DHA with high pressure liquid chromatography (HPLC) for comparison with FT-Raman and FTIR-ATR spectroscopic methods. FT-Raman spectroscopy and ATR-FTIR spectroscopy, alongside chemometric methods using principal component analysis (PCA) and partial least squares (PLS) prediction were shown to be useful techniques to quantify and compare the nectar composition in a range of cultivars of *L. scoparium*.

1. Introduction

The nectar composition of *Leptosperum scoparium* (mānuka), as the plant nectar source for mānuka honey, is of significant interest to the honey industry in New Zealand. The nectar of *L. scoparium* contains the carbohydrate dihydroxyacetone (DHA) which is the precursor chemical for methylglyoxyl (MGO), the unique non-peroxide antibacterial activity (NPA) component of mānuka honey [1–3]. *Leptospermum* honeys are valued for their therapeutic application in wound healing of skin infections [4].

The traditional method of applying the anthrone assay and colorimetric analysis [5] to measure total sugars in *L. scoparium* nectar is a time consuming and laboratory-intensive method. This study presents the potential of attenuated total reflectance (ATR) Fourier transform Infrared (FTIR) and Fourier transform (FT)-Raman spectroscopy combined with the chemometric tool Partial Least Squares Regression analysis (PLSR) to develop a method suitable for the rapid non-destructive discrimination of nectar composition, in particular DHA and saccharide sugars in *L. scoparium*.

Most chromatographic techniques are based on solvent extraction followed by high performance liquid chromatography (HPLC) separation and/or colorimetric and enzymatic analysis [5,6]. Traditional analytical HPLC methods for quantification of analytes of interest require expensive and extensive sample preparation, with additional derivatisation techniques for small molecules such as DHA [7]. FTIR techniques have been shown to be useful in analysing and quantifying sugars in a range of samples such as cereal, baby foods, honey and fruit juices during product processing [6,8–11]. Sultanbawa et al. recently demonstrated that mid infrared techniques provided a good model for predicting methylglyoxyl levels in *Leptospermum polygalifolium* honeys [4]. Infrared spectroscopy is useful in identifying compounds based on the vibration frequencies of their molecular structure and is a sensitive technique for analysing the chemical composition of various sample types [6]. The application of spectroscopic techniques for the quantification of carbohydrates in-situ in plants has received some previous attention from other authors. Past work includes analysis of monosaccharides and polysaccharides applying either FTIR or Raman, and various chemometric analysis techniques to differentiate individual carbohydrates or fingerprint sample types [9]. Raman spectroscopy and FTIR spectroscopy have been used to quantify fructose, glucose, sucrose and saccharose in fruit juices and fermentation stages in vinegar and pineapple juice

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http://dx.doi.org/10.1016/j.vibspec.2016.02.011
0924-2031/

along with quantifying various sugars and acids for grading fruit quality [6,9–11]. Other findings have been reported using FT-Raman to quantify sugars in carrot tissue along with carotenoids and polyacetylene [12–14]. FT-Raman has also been used to analyse the chemical composition of floral honey [15]. Both FTIR and FT-Raman analytical spectroscopy methods are widely applied to identify substances from characteristic spectral patterns ("fingerprinting") and to ascertain quantitatively or qualitatively the amount of a substance in a sample [16,17]. Infrared spectroscopy has been used successfully in a preliminary study on nectar using principal component analysis to distinguish various different plant species nectars [18]. To date there has been no research published using these methods to quantify individual plant nectar components.

2. Experimental methods

The floral nectars of ten different proprietary cultivars of *L. scoparium* were investigated using both attenuated total reflectance (ATR), Fourier transform Infrared (FTIR), and Fourier transform (FT)-Raman spectroscopic techniques, along with high pressure liquid chromatography (HPLC) as a reference analysis. Each cultivar set contained ten replicate plants grown from cuttings of a single parent plant and so were genetically identical i.e. clones of the parent material. Plants were supplied by Comvita New Zealand, and were grown in standard tree and shrub potting mix in 30 cm pots in a glasshouse at the Plant Growth Unit at Massey University, Palmerston North, New Zealand. Nectar was collected in a consistent fashion from 20 flowers from each of the 10 plants of each cultivar and was pooled. Nectar was collected at randomly selected times between 10am and 2pm on any day from flowers at development stage IV [19]. DHA levels in nectar samples were measured using aqueous extraction, derivatisation and analysis by HPLC adapting the method used by Windsor et al. to analyse DHA in honey samples [7]. In addition, DHA levels in nectar samples were validated using a commercial analytical service (R.J. Hills Laboratories Hamilton, New Zealand: http://www.hill-laboratories.com/page/pageid/2145845743/Honey_Testing). HPLC analysis of the sugars fructose, glucose, and sucrose were performed at Institute of Agriculture & Environment, Massey University; Palmerston North, New Zealand.

Three replicates from each nectar sample were used for the development of the spectroscopic methods. ATR-FTIR was used to analyse the same components and quantify using Partial least Squares regression (PLSR), and results and error values were compared to test the accuracy of using FTIR. FT-Raman spectra from a separate nectar set was collected for comparative DHA quantification capability.

2.1. HPLC conditions for DHA analysis

Analyses were performed on a PerkinElmer Series 200 Pump and Auto sampler with a Flexar photo diode array detector (λ = 263 nm). HPLC separations were performed on a Synergi Fusion column (75 × 4.6 mm, 4 μm particle size). The column was heated and held at 30 °C to maintain stable run conditions. Mobile phase A was water: ACN, 70/30, v/v and mobile phase B was 100% ACN. The following 23 min gradient elution was employed: A: B = 90:10 (isocratic 2.5 min), graded to 50:50 (8.0 min), graded to 0:100 (1.5 min), 0:100 (isocratic 7.0 min), graded to 90:10 (1.0 min), 90:10 (isocratic 3.5 min), detection at 263 nm.

2.2. Preparation of reaction solutions

Hydroxyacetone (HA) (3.01 mg/ml) formed the HA internal standard solution. The *O*-(2, 3, 4, 5, 6-pentafluorobenzyl) hydroxylamine (PFBHA) derivatising reagent was 19.8 mg/ml in citrate buffer (0.1 M) adjusted to pH 4 with sodium hydroxide (NaOH) (4 M). DHA (3.88 mg/ml) formed the DHA standard solution.

2.3. Sample preparation

For the preparation of standards, DHA standard solution (100, 80, 60, 40, 20, 10 and 0 μl) was added to tubes 1–7 respectively, and made up to 100 μl with nanopure water. For sample analysis, 20 μl of nectar or standard was pipetted into a mix tube and 25 μl of the HA was added. Derivatisation steps were performed at 25 °C in a controlled temperature room. Each of the HPLC samples and standards was thoroughly mixed and placed in a rack on a rotating Table for 1 h to allow complete dissolution. PFBHA derivatising solution (100 μl) was added to each test tube, which was mixed and placed in a rack on a rotating Table for 1 h to allow for complete derivatisation. Acetonitrile (ACN) (1.5 ml) was added to each test tube and mixed. Nanopure water (0.5 ml) was then added to each test tube and mixed. Samples were then syringe filtered with a 0.22 μm filter into HPLC vials. Vials were placed into the auto sampler and run overnight, and repeat analysis of standards were analysed through each run to check stability of the analysis. DHA calibration curves were generated from tubes 1–7 by linear regression using the HPLC peak area ratios of DHA: HA plotted against the mass of the DHA mass content of the nectar samples were determined against these calibration curves.

Table 1
HPLC data showing DHA and sugar component values for the cultivars normalised to 80° BRIX. Error statistics show standard error of the means. Letters adjacent to column values in superscript indicate the results from the analysis of the comparison of means of each cultivar applying ANOVA with a Tukey pair-wise comparison of the cultivar lines using Minitab statistical software, same letter indicates no significant difference between the cultivars at the 95% confidence level and P-values ≤ 0.05.

Cultivar	Fruct% 80° BRIX	S.E.	Gluc% 80° BRIX	S.E.	Sucrose% 80° BRIX	S.E.	DHA 80° BRIX mg/kg	S.E.
BS	43.22 ^{f}	0.22	35.94 ^{a}	0.19	0.85 ^{b}	0.06	5177.02 ^{b,c,d,e}	677.10
G	44.90 ^{e,f}	0.73	33.24 ^{b}	0.59	1.87 ^{a}	0.20	4116.01 ^{a,b}	521.43
O	46.73 ^{d,e}	0.51	32.82 ^{b,c}	0.49	0.45 ^{c,d}	0.04	5981.54 ^{b,c}	442.12
PU	46.87 ^{c,d,e}	0.27	32.47 ^{b,c,d}	0.25	0.66 ^{b,c}	0.02	2714.35 ^{b,c,d}	380.44
B	46.97 ^{b,c,d,e}	0.45	32.68 ^{b,c,d}	0.41	0.35 ^{c,d}	0.07	6153.12 ^{e}	469.22
R	48.93 ^{a,b,c,d}	0.40	30.78 ^{c,d,e}	0.37	0.29 ^{d}	0.05	7459.07 ^{a,b}	373.56
P	49.25 ^{a,b,c}	0.46	30.55 ^{d,e}	0.40	0.20 ^{d}	0.07	3058.88 ^{c,d,e}	352.04
Y	49.41 ^{a,b}	0.73	29.90 ^{e}	0.73	0.69 ^{b,c}	0.02	4843.71 ^{d,e}	111.35
MG	49.70 ^{a}	0.86	29.79 ^{e}	0.84	0.51 ^{b,c,d}	0.02	3266.70 ^{a}	554.31
LG	50.25 ^{a}	0.20	29.51 ^{e}	0.20	0.24 ^{d}	0.01	4252.20 ^{b,c,d,e}	366.02