



Validity and reliability of Raman spectroscopy for carotenoid assessment in cattle skin

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ABSTRACT

Carotenoids are powerful antioxidants capable of helping to protect the skin from the damaging effects of exposure to sun by reducing the free radicals in skin produced by exposure to ultraviolet radiation, and they may also have a physical protective effect in human skin. Since carotenoids are lipophilic molecules which can be ingested with the diet, they can accumulate in significant quantities in the skin. Several studies on humans have been conducted to evaluate the protective function of carotenoids against various diseases, but there is very limited published information available to understand the mechanism of carotenoid bioavailability in animals. The current study was conducted to investigate the skin carotenoid level (SCL) in two cattle skin sets – weaners with an unknown feeding regime and New Generation Beef (NGB) cattle with monitored feed at three different ages. Rapid analytical and sensitive Raman spectroscopy has been shown to be of interest as a powerful technique for the detection of carotenoids in cattle skin due to the strong resonance enhancement with 532 nm laser excitation. The spectral difference of both types of skin were measured and quantified using univariate and linear discriminant analysis. SCL was higher in NGB cattle than weaners and there is a perfect classification accuracy between weaners and NGB cattle skin using carotenoid markers as a basis. Further work carried out on carotenoid rich NGB cattle skin of 8, 12 and 24 months of age identified an increasing trend in SCL with age. The present work validated the ability of Raman spectroscopy to determine the skin carotenoid level in cattle by comparing it with established HPLC methods. There is an excellent correlation of $R^2 = 0.96$ between the two methods that could serve as a model for future application for larger population studies.

1. Introduction

Skin is a boundary layer between the external environment and the internal body of cells and tissues which offers protection against foreign elements including the ultraviolet (UV) rays from the sun [1,2]. UV exposure induces the formation of free radicals in the skin and has the potential to destroy the cells and damage the skin leading to skin diseases [3]. These free radicals are strong oxidisers of skin and are associated with chronic diseases such as cancer, heart disease and age-related macular degeneration [4]. To neutralise the effect of these free radicals, there is a need for antioxidants capable of reacting with these compounds in skin and protect it from oxidation. This antioxidant

defense system cannot be developed by the body independently, it must come through food rich in carotenoids and vitamins. After digestion of these fat-soluble carotenoids, they can accumulate in many tissues, including the skin's outermost layer which is partially responsible for its colouration [5].

Accumulation of carotenoids varies greatly among animal species and is not fully characterized. It has been reported that dietary carotenoids influence longevity in humans as well as other primates [6,7] and may be essential for proper immune function in cattle [8]. Some researchers have found that β -carotene has demonstrated a positive effect on fertility in cattle; in their study a deficiency in cattle resulted in incidence of silent estruses, less conception rates, higher embryonic

Abbreviations: SCL, skin carotenoid level; NGB, new generation beef; HPLC, High Performance Liquid Chromatography; UV, ultraviolet; LASRA, New Zealand Leather and Shoe Research Association; LDA, linear discriminant analysis; ANOVA, analysis of variance; PCA, principal component analysis.

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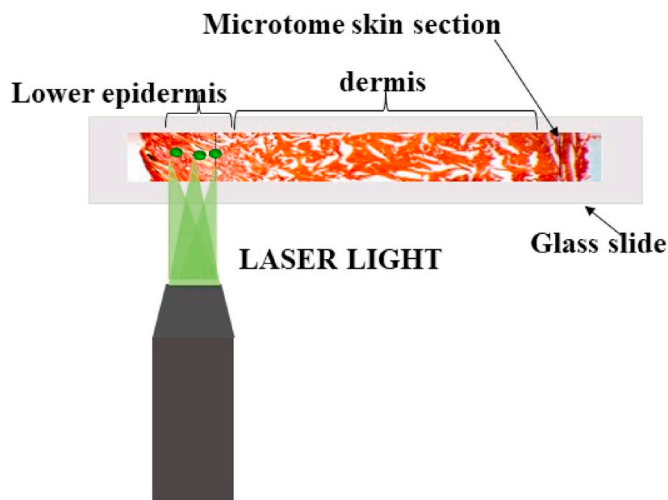


Fig. 1. The region of skin examined by Raman spectroscopy.

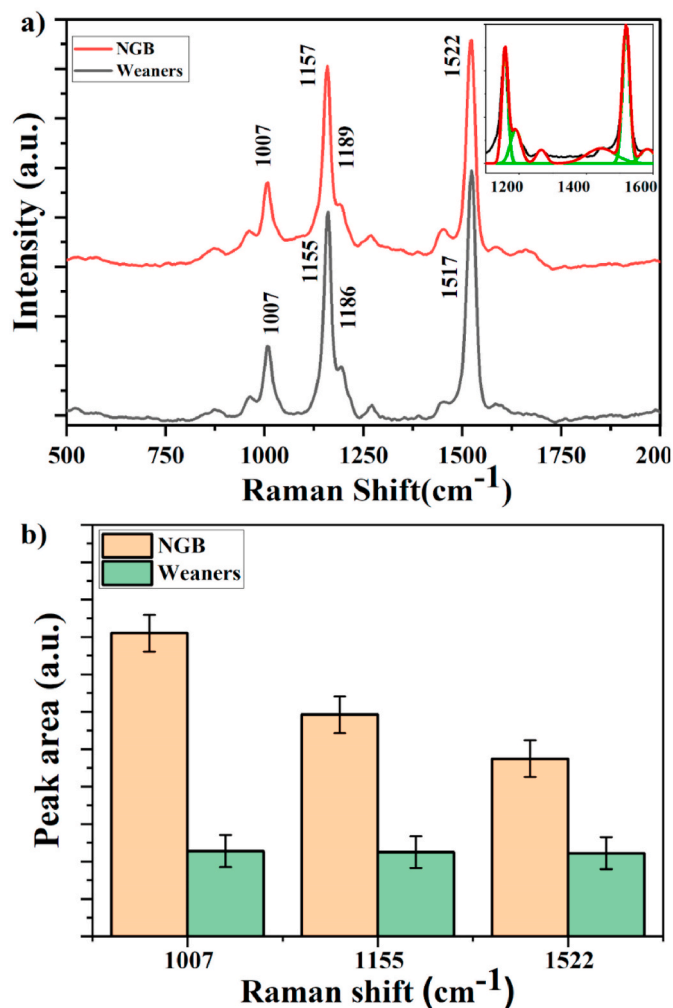


Fig. 2. (A) Average Raman spectra of weaner and 8-month old NGB cattle skin obtained from the lower epidermis (inset: deconvoluted peaks for measuring peak areas) and (B) average peak area of major carotenoid bands (1007, 1155 and 1522 cm^{-1}).

Table 1

Confusion matrix for the classification of weaners and NGB based on the PCA-LDA model.

Groups	Predicted Groups		
	Weaners	NGB	Total
Weaners	9 90%	1 10%	10 100%
NGB	2 20%	8 80%	10 100.00%
Total	11 55%	9 45%	20 100.00%

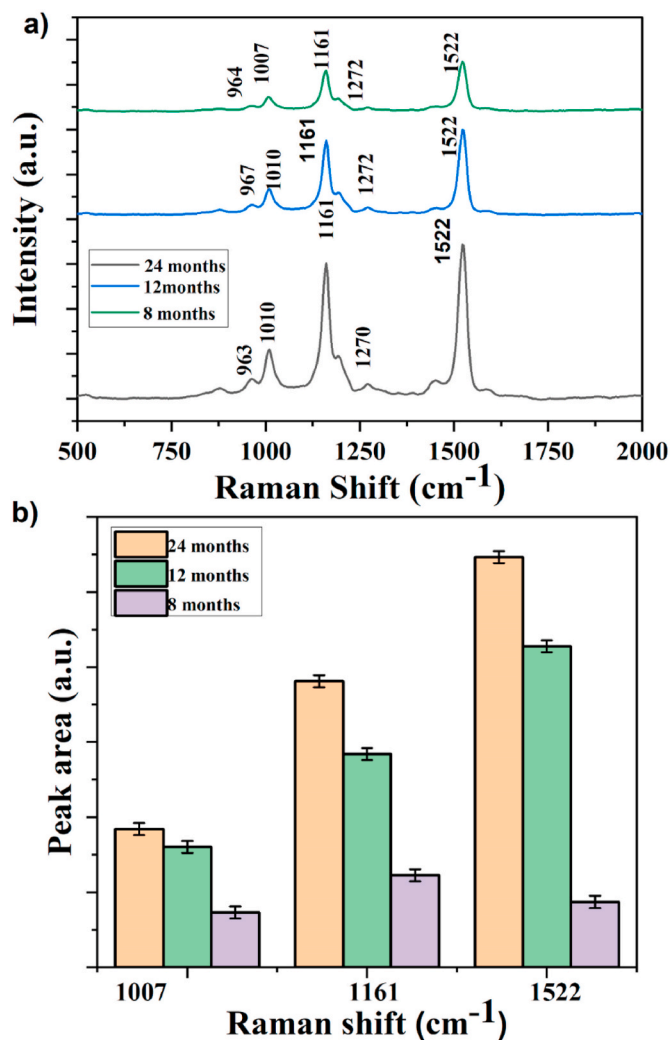


Fig. 3. (A) Average Raman spectra of NGB 8, 12- and 24-months old cattle skin obtained from the lower epidermis and (B) average peak area of major carotenoid bands.

Table 2

Pearson's correlation coefficient (r) for averaged ten skin samples between NGB 8, 12- and 24-months old cattle skin.

Raman shift (cm^{-1})	1007–1011	1161	1522
1007–1011	1	0.68972 ^a	0.91337 ^a
1161	0.68972 ^a	1	0.53133 ^a
1522	0.91337 ^a	0.53133 ^a	1

^a Correlation is significant at the 0.05 level and 'r' value > 0.7 indicates strong correlation.

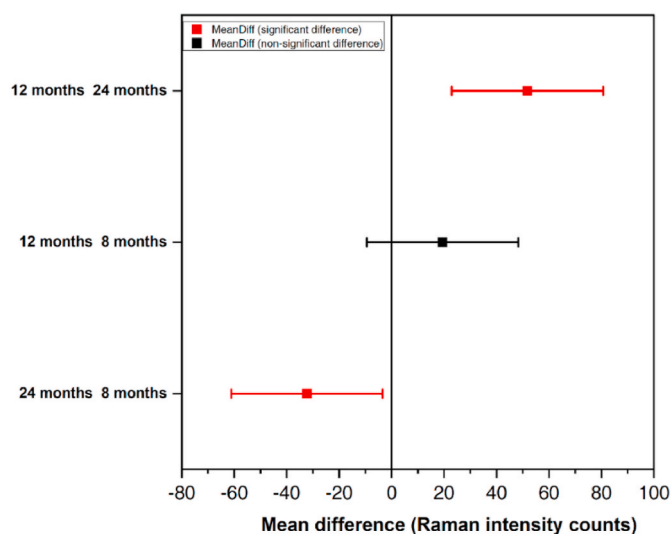


Fig. 4. Mean difference Raman intensities using Tukey Kramer's means comparison test of NGB 8, 12- and 24-months old cattle skin.

death and inferior composition of colostrum [9]. Data on variability in carotenoid concentration data in cattle are limited and may be useful for their management [10] which creates research interest to analyse cattle skin. It has been reported that the higher the dietary intake of carotenoids, the lower will be the risk of bacterial and fungal diseases in animals [11]. Carotenoid studies have been conducted on human skin for age-related diseases [12], or on animals [13], birds [14] or fishes [15] where the carotenoid pigmentation is important for the commercial market.

Hides and skins are a by-product of meat industry and, in turn, generates returns of over a billion dollars from conversion to leather through the global leather industry [16]. Larger countries rely on crops for forage to raise cattle whereas New Zealand relies predominantly on pasture [17].

Grassland-based systems are more environmentally and animal-welfare friendly [18] and can provide a good source of nutrients for animals [19]. Also, recently consumers have become more interested in the origin and method of production of their food, and demand clear information in this regard [20]. Robust methods are required for tracing diet and the age of animal at the time of slaughter for authentication. Carotenoid pigments are potential biomarkers for authenticating carcasses produced from animals fed on different diet [21–23]. Although carotenoid persistence has been studied in both lamb plasma and fat [24], their latency of appearance has received less attention.

There are several types of carotenoids in human and animal skin; including α -carotene, β -carotene, lutein, zeaxanthin, lycopene, lutein, beta-cryptoxanthin; but particularly β -carotene and lycopene, play an important role in protection against photooxidative processes [5,25]. The gold standard used for the detection of carotenoids is High Performance Liquid Chromatography (HPLC) [26,27]. The limitation with this technique is the high cost, extensive extraction protocol which destroys the sample, long sample preparation time and the technique cannot easily be used *in vivo*. Thus, there is a need for an alternative analytical method which requires minimal or no sample preparation, allows the sample recovery after analysis, is quick and has high sensitivity and specificity. Raman spectroscopy is a powerful laser spectroscopic technique that can be used to detect the vibrational energy level of molecules within the sample and gives a spectral 'fingerprint' of the individual molecules. It is considered as a valuable tool for the detection and quantification of carotenoids [28,29]. The positions and intensities of spectral bands [30] can be assessed for the structural analysis of lower epidermis layer of skin. Raman spectral analysis can reveal the biochemical information of the skin with minimal amount of sample,

delivering fast results, resistant to water interference [31], not causing any damage to the sample (with low laser power) and allowing the possibility of in-situ detection. Carotenoids, due to its highly resonant polyene molecular structure [5] is ideal for Raman analysis and when these carotenoids are excited in the visible wavelength range (e.g. 532 nm), behave as an ideal biomarker for Raman analysis because strong resonance results in more efficient scattering and increases the sensitivity by several orders of magnitude [32]. In several studies using reflectance spectroscopy, carotenoids from plasma and adipose tissue were identified as biomarkers of pasture-feeding [33,34].

Microscopic examination of dairy cattle skin demonstrates two structurally different layers (Fig. 1). The upper layer, originally the outer surface of the skin, has hair embedded in the epidermis which is removed. There is a layer underneath called the lower epidermis which forms the grain layer of leather with hair remnants after conventional depilation. The Raman analysis in this study has been performed on the lower epidermis of cattle skin.

This work also provides an important calibration of the Raman intensity of the carotenoid against the analytical data obtained from High Performance Liquid Chromatography (HPLC), which is the gold standard method for determination of carotenoids in the skin. To the best of our knowledge this is the first Raman spectroscopy study on cattle skin to investigate carotenoids in weaners and New Generation Beef (NGB) cattle skin and to study the effect of slaughter age on carotenoid level.

In addition to animal health, traceability of animal production system is a challenge and an increasingly significant interest for scientists and farmers [33,35]. The recent study has shown that dermal carotenoids could serve as marker substances for the entire antioxidant status of cattle skin and prove Raman spectroscopy a reliable and valid method for investigation.

2. Materials and methods

2.1. Sample preparation

Weaner cattle skins aged around 11 months at the time of slaughter were obtained from Venison Packers Feilding Limited, Manawatu, New Zealand. Another set of samples were obtained from Massey University Animal Science Team's New Generation Beef (NGB) project. NGB cattle of Kiwicross X Hereford calves (Kiwicross cattle are a cross breed between Holstein-Friesians and Jerseys) were raised at Keebles and Haurongo Farms near the Massey University Palmerston North campus with approval from the Massey University Animal Ethics Committee. There was regular monitoring of growth rate for these calves. The calves were weaned at 100 kg on to herb mix (chicory, plantain, red and white clover) for the first few months then the calves were put onto pasture feed of perennial ryegrass pasture supplemented with hay/silage until 24 months of age. Skin samples were collected after slaughter in a licensed meat processing facility at 8, 12 and 24 months.

Samples were collected by New Zealand Leather and Shoe Research Association (LASRA®) from the same hind-quarter position on each skin at and stored at less than 4 °C until being prepared for analysis. The samples were sectioned using a Leica CM1850UV Cryostat to 60 μ m thickness. Hairs from the skin were removed by shaving before sectioning the samples. Samples were sectioned laterally to get the lower epidermis. The samples were prevented from drying by continuously spraying water before the measurement to keep them hydrated. Ten skin samples from each category of 8, 12- and 24-months old animals were obtained, and three sections of each skin sample were put onto microscopes slides for Raman analysis.

2.2. Data acquisition and Raman spectral processing

The samples were analysed using a custom-built Raman microscope based on an inverted IX71 Olympus Microscope. A 532 nm excitation laser (with ~10 mW laser power) was focused onto the sample with a

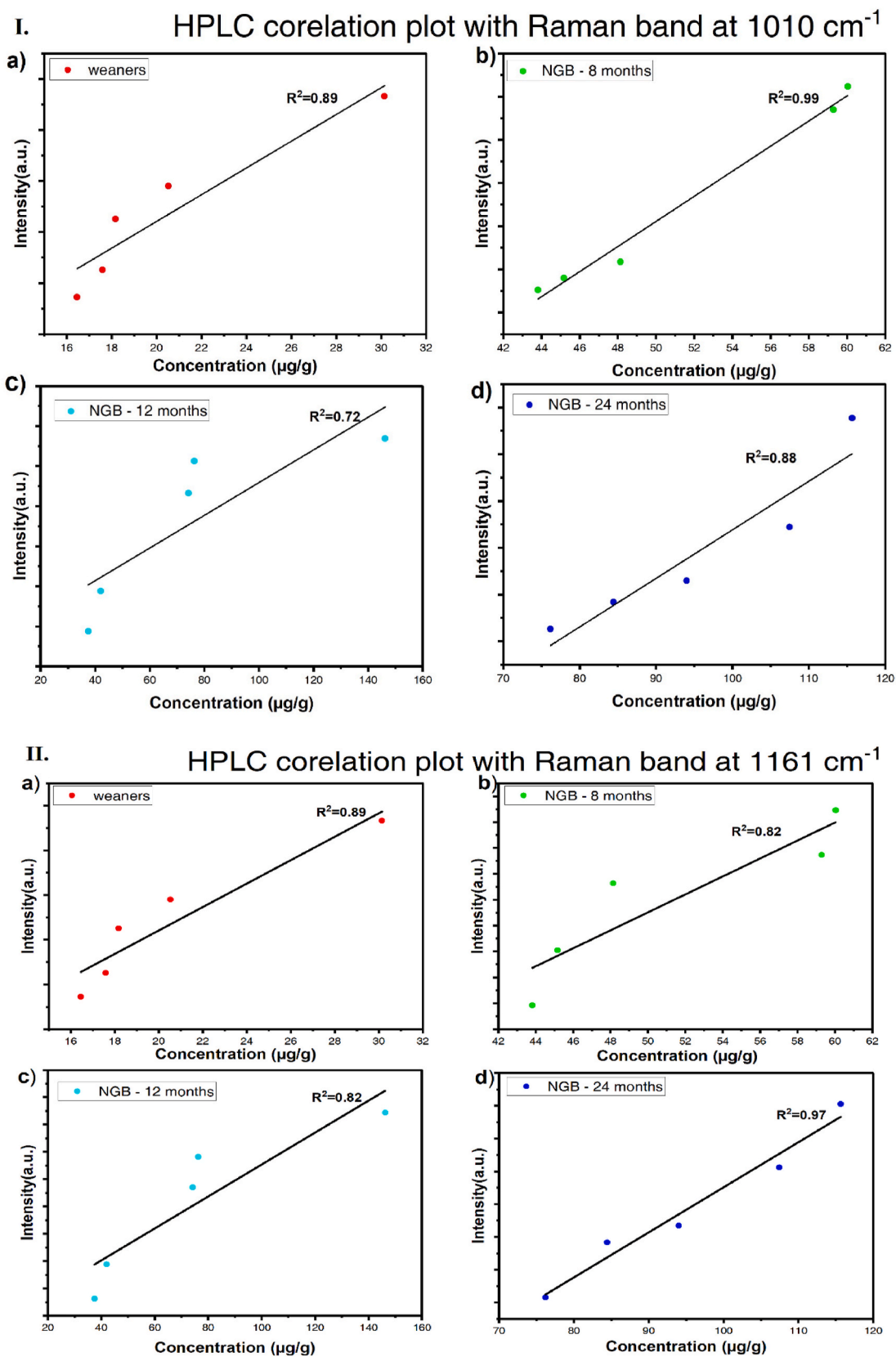


Fig. 5. Calibration curves (a-d) using Raman peak height at (I.) 1010, (II.) 1161 and (III.) 1522 cm⁻¹ and HPLC carotenoid concentration (µg/g) (e) average carotenoid concentration trend; for weaners, New Generation Beef (NGB) of 8, 12, and 24-months old cattle skin.

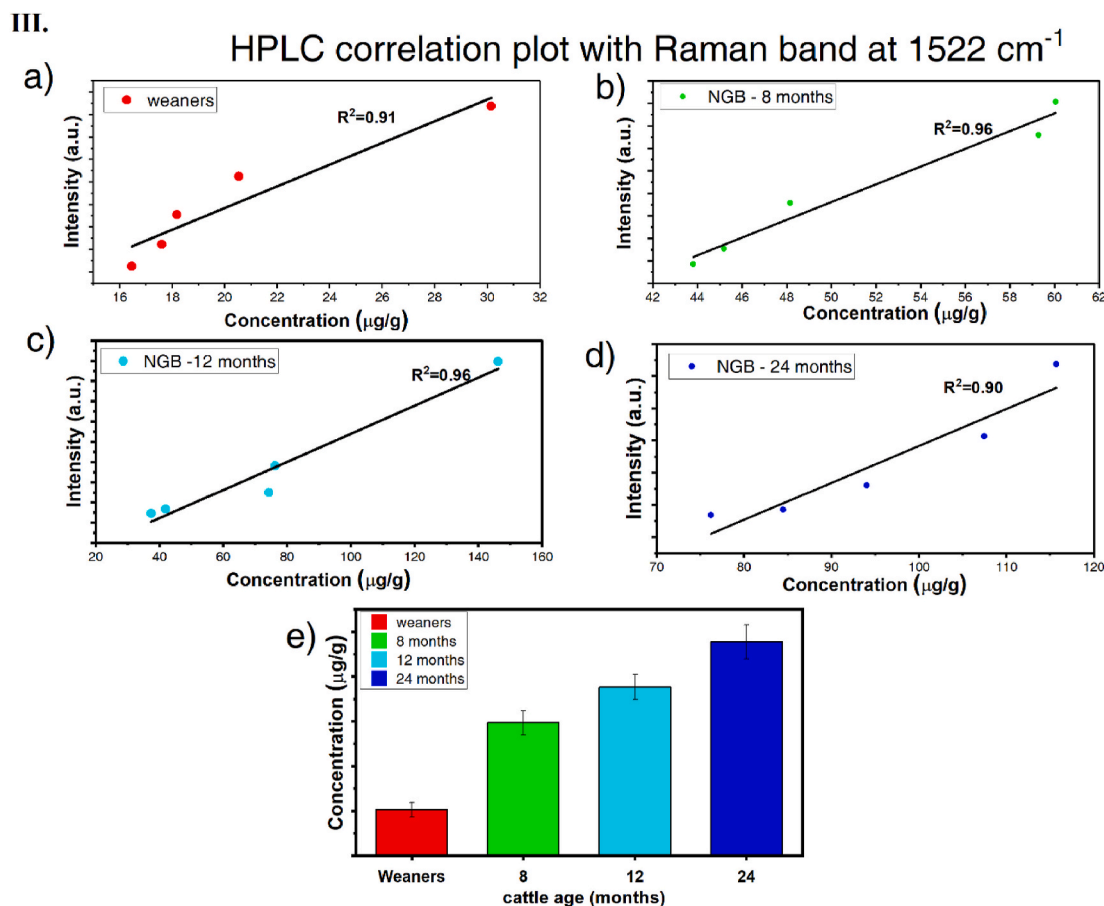


Fig. 5. (continued).

spot size diameter of $\sim 1\text{--}2\ \mu\text{m}$ using $40\times$ magnification and a 0.65 NA objective. A Raman edge filter (12° incident angle) (Iridian Spectral Technologies, Ontario, Canada) directed the excitation into the sample and rejected the Rayleigh scattered light. An additional Raman edge filter (normal incidence) was used to further remove any residual Rayleigh scattering immediately before entering the spectrometer. The Raman scattered light was focussed onto a $50\text{-}\mu\text{m}$ entrance slit of a Teledyne-Princeton Instruments FERGIE spectrometer. Lower epidermis of skin mounted on a glass slide was focussed and imaged using a light microscope.

Triplicates of lateral sections from each cattle skin samples, were used for Raman measurements. Raman spectra were acquired with an exposure time of 5 s per frame for 10 frames (each frame was saved separately). Each spectrum was pre-processed with an algorithm written using the SciKit Learn package [36] in Python 3.7. Baseline correction, background subtraction and average spectra were obtained using the Python algorithm. Then the spectral data was smoothed with five-point Savitzky-Golay smoothing function to smooth spectral noise and normalization was done by dividing each point by the norm of the whole spectrum using Origin 2020b (Origin Lab Corporation, Northampton, Massachusetts, United States). Fitting was performed with Gaussian-Lorentzian functions after subtracting the baseline. The principle of Raman spectrometry is illustrated in Fig. S1.

Raman spectra obtained were analysed using univariate analysis by calculating peak area from fitted carotenoid Raman bands. For classification, Linear Discriminant Analysis (LDA) was employed. Statistical significances were evaluated by analysis of variance (ANOVA) test using Tukey's Means of Comparison test using origin software with significance at a p-value below 0.05 to find statistical difference between means of carotenoid peak intensity values obtained between 8, 12 and

24 months. Calibration curves were created using the peak heights (intensity) of carotenoid bands for univariate analysis.

2.3. HPLC system

To validate the Raman results, the correlation between Raman intensity and high-performance liquid chromatography (HPLC) was measured. One cm diameter disks were punched out of the same area of cattle skin with a gasket punch. The disks were lyophilised on a freeze drier (Labconco, USA). Sections ($60\ \mu\text{m}$ thick) were cut from the lower epidermis of the lyophilised samples using a freezing microtome (Leica CM1850 UV, Germany). Fifteen sections from the lower epidermis of each skin were weighed into microcentrifuge tubes for extraction.

The carotenoid in the sample was extracted with a solvent composed of 20% tetrahydrofuran (BDH Chemicals, New Zealand) in methanol (Fisher Chemical, USA) with 20 mg/L 2,6-Di-*tert*-butyl-4-methylphenol (Roth, Karlsruhe, Germany) as an antioxidant [26]. Each sample was extracted with 1 mL of solvent, facilitated by vortex mixing and 30 min of sonication at room temperature. The mixture was centrifuged at 13,000 rpm for 30 min, then the supernatant containing the carotenoid extract was analysed by HPLC as follows.

The carotenoid content in the extract was separated on an Acclaim C30 column (Thermo Fisher Scientific, USA). The eluent was isocratic 40% isopropanol (Fisher Scientific, United Kingdom) in methanol. Carotenoids were detected by an Ultraviolet/Visible detector (Thermo Scientific, DAD 3000, USA) at a wavelength of 450 nm. β -Carotene (Sigma-Aldrich, USA) dissolved in the extraction solvent and diluted to adequate concentrations was used as the calibration standard.

3. Results and discussion

3.1. Optical microscopy

Polarised light microscopy was used to examine the lower epidermis and dermis of cattle skin [37]. Fig. 1 shows dermis of skin which is made up of large collagen fibre bundles and the lower epidermis, also called stratum basale, which is the deepest epidermal layer and below which lies the different layers of dermis. The cells in lower epidermis bond to the dermis via intertwining collagen fibres [38]. The lower epidermis was investigated throughout the study.

3.2. Skin carotenoid level of weaners and NGB cattle skin

In our previous work, we have examined the different layers of skin and found carotenoids predominantly in the lower epidermis [39]. Therefore, as an extension to our previous work, the current study is based on Raman measurements of the lower epidermis of weaners with an unknown feeding regime and NGB skins with a known diet.

Ten samples were analysed from each weaner and NGB cattle skin sample to identify any variation in carotenoid levels and develop a relationship between cattle group and skin carotenoid Raman status. The average Raman spectra of lower epidermis of skin of weaner and NGB cattle skin is shown in Fig. 2.

The results show that carotenoids are a very strong Raman scatterer with very high enhancement and no fluorescence emission. This is due to the highly conjugated structure of the carotenoids with alternate single and double carbon chains, methyl side chains and different end groups [26]. This enables the detection of carotenoids with ease even with complex biological systems. Raman spectra were acquired under strong resonance conditions with the carotenoids. The resonance effect selectively enhances Raman scattering from the carotenoids due to the strong and narrow carotenoid absorption spectrum [40]. Fig. 2a Demonstrates that there are three major Raman bands in the analysis of carotenoids [41,42]. The Raman peak at 1522 cm^{-1} is due to the stretching vibrations of the conjugated C=C backbone of carotenoids and is sensitive to differences in carotenoids molecular conformation. The vibration band at 1155 cm^{-1} is attributed to C-H in-plane bending and C-C stretching vibrations of the polyene chain. The vibration band at 1007 cm^{-1} arises from C-CH₃ in plane-rocking deformations of methyl side chains coupled to C-C bonds. Weaners and NGB skin samples have 1155 and 1522 cm^{-1} sensitive molecular environments whereas 1007 is insensitive to any change, meaning that a carotenoid found at this characteristic Raman peak has a similar spectrum in both categories irrespective of the different set of animals [43]. This is an important observation to assign specific carotenoid Raman signature at 1522 cm^{-1} to Lutein for NGB and 1517 cm^{-1} to β -carotene for weaner skins. Such sensitive spectral variation illustrates the ability to distinguish carotenoids by the position of this vibration [44]. It was supported by the literature where carotenoids are classified into two types – oxygen deficient carotenes, and oxygen rich xanthophylls. The carotenes are nonpolar hydrocarbons and include β -carotene, α -carotene, and lycopene. The xanthophylls have hydroxyl or keto end groups and are thus more polar compounds, including lutein, zeaxanthin, canthaxanthin, and β -cryptoxanthin [45]. Lutein is the most dominant carotenoid [46] found in animal food and is predominant over carotenes which are not selectively absorbed whereas β -carotene is predominant in diet-derived from milk [47,48]. The detection of β -carotene and lutein spectral peak in cattle skin differentiates the two groups and may serve as a characteristic marker identified using Raman spectroscopy.

Distribution of Carotenoids in weaners and NGB cattle skin.

Spectral variation between weaners and NGB cattle skin is not significant for quantification, therefore, detailed analysis of the above-mentioned spectral regions was done with deconvolution of the spectra of all three prominent carotenoid peaks (Fig. 2a inset). The analysis was carried out using the “quick peaks from peak analyser” tool

of Origin software. For peak quantification, peak area from normalised spectra were calculated from fitted peaks of major carotenoid Raman bands 1004 , 1155 and 1522 cm^{-1} . The main aim of Raman peak fitting is to determine the peak parameters such as peak area, as accurately as possible. Peak intensity, one of the parameters, is not considered as it is most of the times affected by the noise and difficult to measure with precision [49].

The distribution of carotenoids in cattle skin samples, measured by Raman spectroscopy, in Fig. 2b Shows that total carotenoid content in NGB cattle skin is 3-fold higher than weaner cattle skin (Table S1). Since wave number 1003 cm^{-1} has contributions from phenylalanine as well as carotenoids, the extent of change in this peak intensity was higher than the change seen in wavenumbers 1156 and 1524 cm^{-1} .

To support the univariate data, supervised method, linear discriminant analysis (LDA) was used. This assumes the existence of classes and then proceeds to constructs a function (the discriminant) that gives the best separation between the classes [50]. It shows how well the classes are separated as well as where the classification fit is robust and where it is misinterpreted. The principal components from the principal component analysis (PCA) can also be used to construct the discriminant function in LDA (PCA-LDA). LDA assumed that the data was Gaussian distributed, that all rows must belong to one group (samples are mutually exclusive) and that the variances are the same for both groups. When LDA is done on the PC scores, the mean centre of each grouping is calculated, and each spectrum is predicted to belong to one of the groups based on its distance from the centre of the group. The accuracy of the prediction is an indication of how well the groups are separated. The classification summary in the form of confusion matrix is shown in Table 1.

The Wilk's Lambda test was conducted on the discriminant variable and found that the discriminant function is highly significant ($p < 0.05$) in agreement with the classification summary. The cross-validation summary table shows that weaners has a classification accuracy of 90% and NGB has 80% which proves that both are mutually exclusive.

3.3. Carotenoids and skin aging

Several human studies have been performed in the past relating the individual age with carotenoids [51], but this is the first-time cattle age is investigated using the carotenoid biomarker with a sensitive technique, Raman spectroscopy. It was established and reported in past studies that younger individuals have higher carotenoid content in their skin as compared to the older ones with the loss of major nutrients from the body due to diseases, skin aging, and less availability of anti-oxidants that weakens the defence system of the body [52] whereas this has never been explored for animals. With this objective, a study is designed to investigate total carotenoid content in cattle skin of different ages fed on pasture before slaughter. Skin of 8 months, 12 months, and 24 months old NGB cattle skin was analysed using Raman spectroscopy.

Fig. 3a Shows the average Raman spectra of NGB cattle skin of different age with three major carotenoid bands. On visual examination, there was no spectral differences observed in the carotenoid content among three age groups but there is a significant variation in the skin carotenoid status after performing univariate analysis on the peak areas of prominent carotenoid peaks. Fig. 3b Shows the 1007 or 1010 cm^{-1} carotenoid peak has 34% increase in carotenoids from 8 months to 12 months with a further increase of 20% in 24 months. Similarly, 1161 cm^{-1} also showed an increasing trend of 35% from 8 to 12 months, then 20.2% increase at 24 months whereas 1522 cm^{-1} has a maximum increase of 70% from 8 to 12 months, then 40% at 24 months.

Statistical analysis was carried out on the Raman spectral results using analysis of variance (ANOVA) test. For each wavenumber analysed (1007 , 1161 , and 1522 cm^{-1}) post hoc Tukey–Kramer means comparison test was conducted to identify the significant difference between the groups. Pearson correlation coefficient and significance differences were considered significant at a p-value below 0.05.

Pearson's correlation was employed to test the strength of correlation of the spectra of ten skin samples of each age groups. The average spectra obtained for different age groups correlated with each other with a "r" value close to 1. (Table 2). There was a significant effect of age on the obtained values at $p < 0.05$ level for the three age groups ($p = 0.0019$). This demonstrates that the spectra between three age groups were significantly different.

For each pair of groups, the graph shows an estimate for the difference of means and the Tukey-adjusted 95% confidence intervals for the difference. Intervals that contain 0 indicate that the difference of means is not significant. Intervals that do not contain 0 indicate significant differences. Tukey's Means of Comparison test has revealed further information regarding relationships between age groups and it was found that 24 months is significantly different from other age groups (Fig. 4.) with the smallest means.

3.4. Raman spectroscopy method correlated with HPLC analysis

To validate the performance of Raman spectroscopy, we have used the gold standard HPLC technique for skin carotenoid analysis. HPLC measurements (Table S2) were carried out on weaners and NGB cattle skin of different ages. (8 months, 12 months and 24 months). For perfect validation of Raman carotenoid results with HPLC without any possible interferents, ultraviolet absorbance scan of carotenoid external standard over a range of wavelengths from 245 nm to 600 nm was obtained which perfectly matched with the extracted carotenoid (Fig. S2). The peak intensities of major carotenoids Raman bands at 1010, 1161 and 1522 cm^{-1} was used for validation with concentration of carotenoids obtained from HPLC results. Comparing the two datasets, we are aware of systematic errors where HPLC analysis is designed to 'see' a definite subset of species in the entire depth of microtome samples whereas Raman effect pick up C=C signal from carotenoid molecules with highly resonant Raman signal. Despite these unavoidable errors, we have obtained the best correlation between Raman and HPLC data for carotenoid peak at 1522 cm^{-1} in all age groups, as observed from Fig. 5, with highest R^2 value 0.96 for 12 months. Raman band at 1010 cm^{-1} has moderate correlation with HPLC data for NGB 12 months with R^2 value 0.72. Significant differences were observed in the intensities of major carotenoid bands of two different fed cattle skins with p -value < 0.001 .

These results validate Raman spectroscopy as an accurate and objective method for animal skin carotenoid measurements.

4. Conclusions

Raman spectroscopy has an excellent sensitivity for detecting carotenoids, because of the strong spectral signatures of the conjugated C=C and C=C functional modes. The study carried out on weaners and NGB cattle skin using carotenoids as a biomarker is highly promising. Univariate and linear discriminant analysis methods were used for cross-validating Raman results with HPLC data. There is very good correlation between the two different methodologies. The results obtained shows that Raman spectroscopy is well-suited to determine the antioxidative status in animal skin, selectively and sensitively. Skins of weaners had a lower carotenoid content compared to monitored pasture fed NGB cattle skin. Another observation was that older NGB cattle skin demonstrated a higher carotenoid content than the skins from younger animals. The validation results will make it possible to closely correlate both methods – Raman and HPLC in justifying the common conclusion after skin carotenoid analysis. This study helps in assessing the skin, identifying ways for improving the animal skin and protect it from diseases [11].

Author statement

Megha Mehta: Conceptualization, Methodology, Software, Visualization, Data curation, Formal Analysis, Investigation, Writing – Original draft preparation. Rafea Naffa: Methodology, Resources, Writing –

Reviewing and Editing. Wenkai Zhang: Data curation. Nicola M. Schreurs: Resources. Mark Waterland: Resources, Writing – Reviewing and Editing. Sue Cooper: Writing – Reviewing and Editing. Geoff Holmes: Funding acquisition, Resources, Writing – Reviewing and Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2021.101036>.

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