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Abstract. For a long time, the antioxidative status in cattle has been discussed as an indicator for stress conditions resulting from disease or exertion. Until now, invasive approaches have been necessary to obtain blood samples or biopsy materials and gain insights into the antioxidative status of cattle. Due to these efforts and the costs of the analyses, serial sampling is feasible in an experimental setting, but not for measurements on a routine basis. The present study focuses on the feasibility of an innovative, noninvasive spectroscopic technique that allows *in vivo* measurements of carotenoids in the skin by reflection spectroscopy. To this end, in a first trial, repeated measurements of the carotenoid concentration of the udder skin were performed on 25 healthy cattle from different breeds. Carotenoid concentrations showed highly significant differences between individual animals ($P < 0.001$), although they were kept under the same environmental conditions and received the same diet. The carotenoid concentrations in "sensitive" and "robust" cows (evaluated by a temperament test) differed significantly ($P < 0.005$), with higher concentrations observed in robust cows. [DOI: 10.1117/1.JBO.17.10.101514]

Keywords: antioxidants; β-carotene; cattle; cow; temperament; *in vivo*.

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1 Introduction

The effect of carotenoids on health and productivity in dairy cattle, as well as on the properties of dairy products, has been the focus of scientific interest for a long time. Carotenoids give dairy products a yellowish stain, which consumers relate to a healthy product. Various disorders of cattle such as mastitis, fatty liver syndrome, and infertility have been demonstrated to be associated with low plasma carotenoid concentrations.[1,2] The latter phenomenon might be a reflection of oxidative stress,[3,4] which occurs when the balance between the reactive oxygen species (ROS) and the antioxidative protective mechanisms of the organism is shifted in favor of the ROS.[5,6] Such conditions form a potential hazard for both the well-being of the individual animal and its productivity. Endogenous generation of ROS and RNS (reactive nitrogen species) in the body is inevitable. ROS and RNS, however, are capable of destroying biologically important molecules, e.g., DNA, proteins, carbohydrates, and lipids.[7] Therefore, body cells dispose overprotective systems to avoid oxidative damage. These systems comprise various enzymatic and nonenzymatic antioxidative mechanisms.[8] Antioxidants have been shown to act synergistically, by forming a protective chain, thus protecting each other against destruction induced by free radicals and other reactive species.[9,10] One important group of antioxidants are the carotenoids, which can react with free radicals and in various ways become effective as antioxidants.[11–13] Owing to these interactions, the carotenoids could represent marker substances for the entire antioxidative protective system of the organism.[3,14]

In bovine tissues, among all carotenoids, only β-carotene can be found at higher concentrations.[15] In addition, 13-*cis*- and all-*trans*-β-carotene and lutein have been detected in the plasma and milk of bovines.[16,17] Neither humans nor animals are capable of *de novo* synthesis of carotenoids and therefore depend on dietary supply.[18,19] Until now, high-performance liquid chromatography (HPLC) has been widely used in veterinary medicine for the analysis and quantitative determination of carotenoids.[20] Today, the determination of β-carotene in blood samples is applied on a routine basis in herd health-monitoring programs. Determination of the carotenoids by HPLC requires sampling of either blood or biopsy materials. Because of the invasive character of the sampling procedures and the costs for the analyses, the latter approach is not suitable for serial analyses.[21]

In humans, several studies have been undertaken in recent years to detect antioxidants, in particular β-carotene and lycopene, in the skin using an optical method based on resonance Raman spectroscopy, which has been demonstrated to deliver fast results, being noninvasive.[22] Dermal carotenoid concentrations as determined in human volunteers yielded a wide range of levels.[23,24] The latter findings were related to differences in the digestion and metabolism of dietary carotenoids, which could be genetically dependent,[25] based on eating habits, lifestyles and, possibly, different stress conditions of volunteers.[26] In humans, dermal oxidative stress has been demonstrated following sun irradiation,[11,27–29] environmental hazards[30], illness or inflammation, and various stress conditions.[26,31] Raman spectroscopic measurements are also used in the field of different food products like meat, eggs, and products produced from milk.[32,33] These measurements permit quantitative and qualitative determinations of carotenoids.[34,35]

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β-Carotene levels in cattle have successfully been determined by Niedorf[36] in biopsies obtained from udder skin using reflection spectroscopy. A close correlation of the results was found with those of comparative HPLC measurements ($R = 0.711$).[36] Applying spectroscopy allows higher numbers of individuals to be examined, as well as investigation of the efficacy and kinetics following dietary supply of carotenoid antioxidants and their accumulation in the skin, *in vivo*[26,37–42] and *in vitro*.[43]

The purpose of the present study was to apply spectroscopic measurements to monitor the carotenoid concentrations in the skin of cattle by using an innovative, noninvasive technique including a handheld miniaturized spectroscopic system (MSS).

2 Materials and Methods

2.1 *Study Design*

The study consisted of two different trials. Experiment 1 aimed at the noninvasive *in vivo* determination of carotenoid concentrations in the bovine udder skin, and experiment 2 included repeated determinations of the carotenoid concentrations in the skin of the same animal.

2.1.1 *Experiment 1*

Twenty-five clinically healthy female cattle, aged between 2 and 11 years, were included in the trial. Twenty cows were of the breed German Holstein and 5 were of the breed German Red Holstein. All animals were kept under the same conditions and were fed at need with a ration consisting of grass silage, hay, and concentrates. The lactation stadium, however, differed individually among the cows.

Noninvasive *in vivo* carotenoid measurements were performed on three pre-determined sites (A, B, C) on the surface of the udder, each located 10 cm proximally to the teat basis. Two sites (A, B) were located perpendicular above the teats, and the third site (C) was located halfway in a direct line between points A and B, as shown in Fig. 1. Each site was subjected to three subsequent measurements. To this end, the MSS was removed from the skin between measurements and then positioned once more on the same skin area. The time interval between the measurements was only a few seconds. Prior to the measurements, the respective skin area was shaved with a disposable razor and the different sites were marked. All measurements were performed on the left side of the udder.

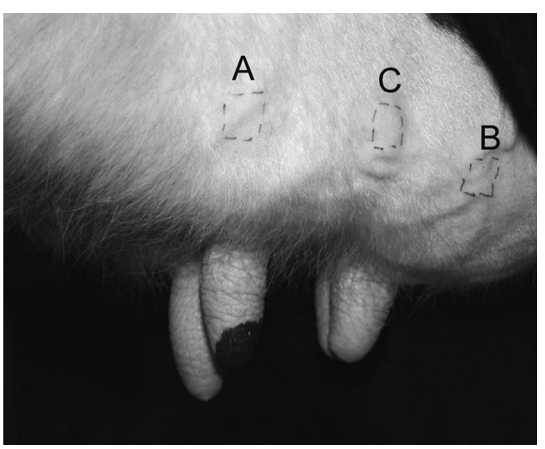

Fig. 1 Measuring sites on the udder skin of a cow.

2.1.2 *Experiment 2*

Thirteen cows aged between 3 and 7 years were included in the two parts of experiment 2. Ten animals (9 of the breed German Holstein and 1 of the breed Jersey) were used in part 1. Twelve cows (10 of the breed German Holstein, 1 of the breed German Red Holstein, and 1 of the breed Jersey) were used in part 2. All cows were kept under the same conditions and were fed at need with a ration consisting of silage, hay, concentrates, and straw. During the test period, the animals had not been exposed to any experimental manipulations or any other types of stress; cow were not pregnant, nor had they calved within the last 6 months. No drugs had been administered to the animals during a 3-month period preceding the trial. The carotenoid measurements in the frame of this study were performed as described previously.[44]

In the part 1, three measurements were performed on 10 animals within four days. Subsequently, in part 2, 12 cows were evaluated as either "sensitive" ($n = 6$) or "robust" ($n = 6$) according to the results of a behavioral test of the temperament of the cattle.[45] The ratings were 1 = calm, no movement; 2 = slightly restless; 3 = squirming; 4 = continuous, very vigorous movement; 5 = rearing, twisting of the body and struggling violently. Scores 3, 4, and 5 were categorized as nervous behavior. To verify the objectiveness of evaluation of the temperament test, the 25 animals from experiment 1 were judged by two independent persons simultaneously. The coefficient of concordance W was calculated. In addition, stress indicators like tail strokes, attempted escapes, uncoordinated reciprocating movement of the head, calls, urination, and defecation[46–50] were recorded. All six animals categorized as being sensitive exhibited at least three stress indicators, whereas animals categorized as being robust exhibited fewer than three stress indicators. The two cows of the breeds German Red Holstein and Jersey belonged to the group of the robust cows, whereas the group of the sensitive cows consisted only of German Holstein breed cows.

2.2 *Miniaturized Spectroscopic System*

For the noninvasive determination of the carotenoid concentration in the bovine udder skin, an LED-based MSS (Opsolution GmbH, Kassel, Germany) was used. The measuring principle of the MSS is based on reflection spectroscopy. Figure 2 shows the $-\log 10$ of the diffuse reflected spectrum obtained in vivo from the bovine udder skin. The form of the reflection spectrum is determined by the presence of dermal chromophores. Taking into consideration the absorption spectrum of carotenoids, which lies in the blue-green range of the optical spectrum,[22] the blue LED-emitted bright spectrum in the range between 440 and 490 nm was used as a source of excitation. The small dip in the reflected spectrum, measured in the range between 458 and 472 nm, is associated with the absorption of carotenoids in the skin sample (see Fig. 2). The value of this dip was recalculated with the relative carotenoid concentration (arbitrary units [a.u.]).

Before *in vivo* measurements were started, the MSS was calibrated *in vitro* using bovine skin specimens by the use of resonance Raman spectroscopy, which is specific for the measurement of dermal carotenoids.[34] For this purpose, an area of approximately 0.8 cm^2 was marked on the perimeter with permanent marker, and the center of this area was further used for both reflectance and Raman measurements. The intensity of the

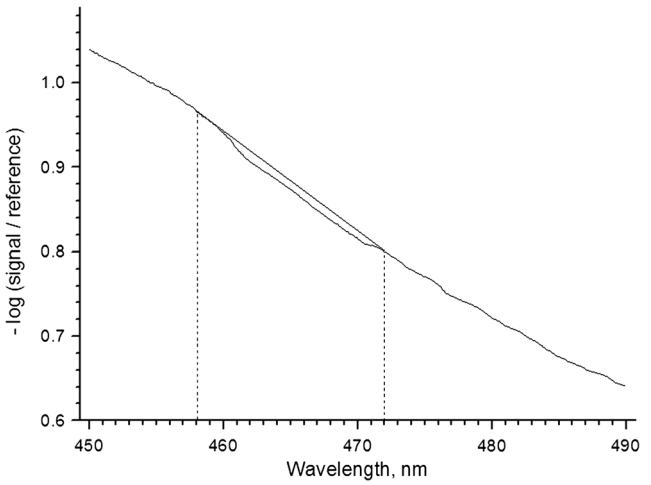

Fig. 2 Diffuse reflectance spectrum obtained in vivo from bovine udder skin under LED excitation at 465 ± 25 nm.

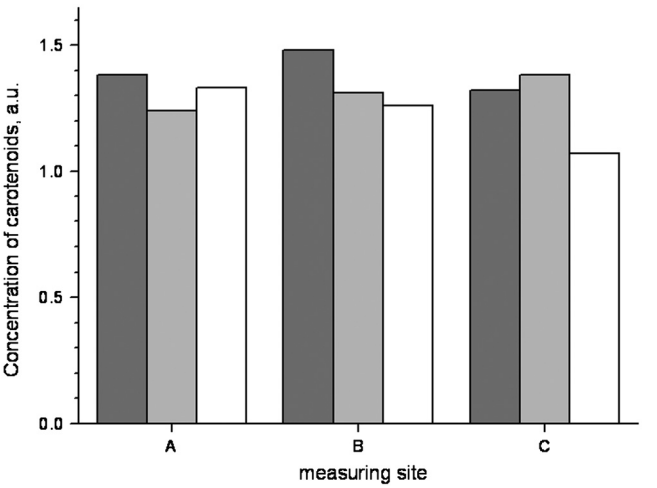

Fig. 3 Carotenoid concentrations obtained with the MSS by serial measurements from the three different sites (A, B and C) on the udder skin of an individual animal.

prominent Raman peak at 1525 cm^{-1}, originating from the carbon–carbon double bond stretch vibration of the backbone of carotenoid molecules under the excitation at 488 nm, was analyzed and compared to the size of the dip in the reflectance spectrum. To exclude the influence of light from outside, the optical measurements were performed under full contact between the skin surface and MSS and Raman devices. A strong linear correlation ($R^2 = 0.81$) was achieved. The spread of the measured with the MSS concentrations of carotenoids did not exceed 10% on bovine udder skin *in vitro*. A detailed description of MSS has been previously presented by our group.[44]

2.3 *Statistical Analysis*

Statistical analysis was carried out using the statistics software package SPSS 18.00 for Windows (IBM, Armonk, NY). The data were tested for normal distribution and, as the latter were partially distributed abnormally, the median was used. The Mann–Whitney *U*-test was applied for independent measurements using two samples, whereas the Friedman test was used for dependent measurements using more than two samples. The Kruskal–Wallis test was applied for comparing more than two different groups.

To evaluate correlations between the values, the Spearman correlation test was used. Values with $P < 0.05$ were considered statistically significant.

3 Results

3.1 *Noninvasive In Vivo Determination of Dermal Carotenoids in Bovine Udder Skin and Reproducibility of Measurements (Experiment 1)*

Figure 3 shows the results of the measurements on three different sites (A, B, C) on the udder skin of the same animal. The measuring accuracy of the MSS was determined for each animal by calculating the percentage standard deviation from all three measurements performed on the sites A, B, and C. As a result, a measuring accuracy of 12% was established for the MSS applied to the same area of the skin. In addition, the percentage variation of the carotenoid concentrations as determined on three different sites (A, B, C) was determined for each animal. As a result, a mean variation of 17% was determined for the carotenoid values on the different areas of the specific udders.

Carotenoid concentrations of the same skin areas showed highly significant differences between individual animals ($P < 0.001$) (Fig. 4). The carotenoid concentration in the skin is represented as median ± standard deviation.

The median ($n = 0.25$) of the relative concentration was 0.88 ± 0.25 a.u. The variation range was 1.06 a.u. A factor of 4 could be revealed between the maximum and the minimum value (Fig. 4). The mean standard variation amounted to 0.05 a.u. The correlation between the daily milk yield and the carotenoid concentration was weak ($R^2 < 0.2$).

As only one animal was <3.5 years old, no comparison was made between young and old cattle. Due to the low number of animals, the dependence of the carotenoid concentration on the breed was not computed, either.

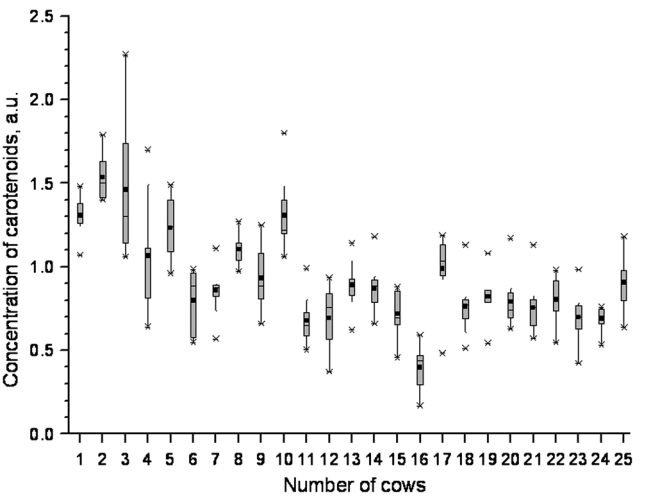

Fig. 4 Carotenoid concentrations measured by MSS and expressed in arbitrary units on the udder skin of 25 cows (median± standard deviation). Cows 1 to 5 were of the breed German Red Holstein and cows 6 to 25 of the breed German Holstein.

3.2 *Carotenoid Concentrations in the Udder Skin of Cattle Kept under Standardized Conditions (Experiment 2)*

Ten healthy cows after undergoing three measurements on the same site of the udder skin within four days exhibited no significant differences in dermal carotenoid concentrations (Fig. 5). During these four days, a mean deviation of 10% was measured for the carotenoid concentrations.

To evaluate differences between observers with respect to the temperament test, the 25 cows from experiment 1 were judged by two persons, independently. The results did not show any significant differences. Using the temperament test, the 12 animals could be clearly categorized as either sensitive ($n = 6$) or robust ($n = 6$). The carotenoid concentrations of these animals are given in Fig. 6.

The difference between the medians of the two groups was significant at $P < 0.05$. The general carotenoid skin udder levels (median of $n = 6$) of the robust cows amounted to 1.11 ± 0.24 a.u, whereas the sensitive cows exhibited a general carotenoid skin udder level of 0.72 ± 0.10 a.u.

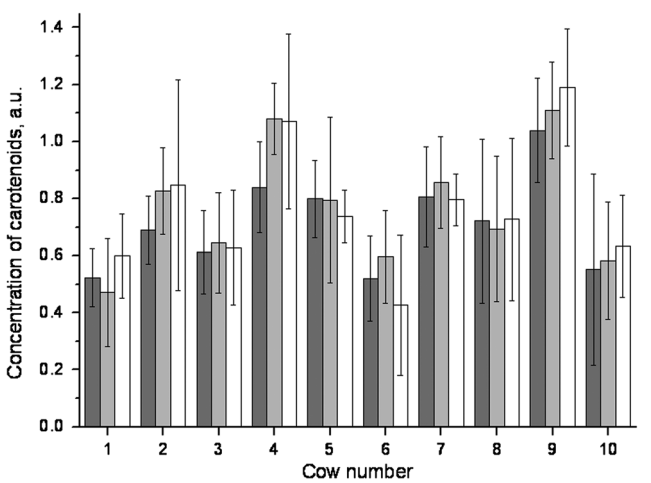

Fig. 5 Carotenoid concentrations on the udder skin of 10 healthy animals determined on four consecutive days. Dark gray column = day 1; light gray column = day 2 or day 3; white column = day 4.

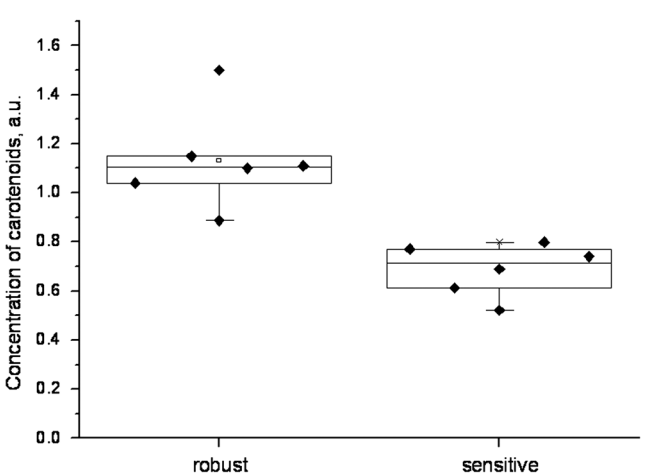

Fig. 6 Median dermal carotenoid concentrations as expressed in arbitrary units measured on the udder of animals evaluated as either sensitive ($n = 6$) or robust ($n = 6$) according to the results of a temperament test (median ± standard deviation).[45]

4 Discussion

The MSS technique, applied in the present study to determine dermal carotenoid concentrations, is suitable for noninvasive measurements of carotenoids in the bovine udder skin. As carotenoids have been demonstrated to represent possible marker substances for the entire antioxidative status in humans,[3,26] this noninvasive measurement opens up great perspectives for the use in cattle, to investigate the oxidative stress caused by high milk yield, illness, or environmental stressors.

In accordance with observations in humans, the present study demonstrates a huge variation in carotenoid levels as determined by MSS measurements in different cattle. Lotthammer and Ahlswede[51] likewise demonstrated significant differences between different individuals in the concentrations of β-carotene in blood plasma. Noziere et al.[16] found that the β-carotene concentrations in the blood plasma of cattle fed with grass silage varied strongly between individual animals, whereas this variability was considerably less profound when the animals were exclusively fed hay. Hesterberg et al.[33] showed by the use of resonance Raman spectroscopy that the concentration of carotenoids in the yolks of hen eggs is also influenced by nutrition and housing conditions. Experiments carried out by Niedorf[36] on isolated perfused udder skin, using reflection spectroscopic measurements, revealed huge variations in the dermal carotenoid concentrations of organs from different individuals.

The animals included in experiment 1 differed regarding their age, breed, and stage of lactation. Calderon et al.[17] ascertained that during the nonlactating phase as well as during the first week of lactation, a reduction occurred in the carotenoid concentration of the blood, which gradually increased during a period of 3 months. Similar observations have been reported by various authors.[52–54] The influence of age on β-carotene concentration in blood is largely unknown. Katsoulos et al.[54] observed significantly higher β-carotene concentrations in younger animals, while—on the contrary—others had observed either higher concentrations in older cattle[55,56] or no age-related differences whatsoever.[57,58] A variety of studies revealed breed-related differences with respect to β-carotene concentrations in the blood.[56,58–61]

Tian et al.[62] showed statistically significant differences in the β-carotene concentration in fat tissues and in color of subcutaneous fat of cattle representing various genotypes. A negative relationship was reported between β-carotene status and stearoyl-CoA desaturase activity in cattle, but a positive relationship existed when cattle were fed on the same diet. For this reason, differences in β-carotene concentrations were related to genetic differences in intestinal dioxygenase being able to metabolize β-carotene to vitamin A.[62] Investigations on human skin also resulted in strong differences regarding β-carotene and lycopene concentrations as determined on the same site in different volunteers.[63,64] In human skin, the distribution and concentrations of carotenoids strongly depend on the state of health of the respective volunteer, his nutritional habits, lifestyle, the investigated area of the body, and the effects of stress factors.[26,34,41]

Serial measurements on the same individual animal revealed no significant alterations in the dermal carotenoid concentrations during four consecutive days, demonstrating hardly any day-to-day variation over a short period of time. Consequently,

the measured values remained relatively stable for each animal under standardized conditions.

This could also be verified by measurements in humans. Darvin et al.[26] demonstrated that dermal carotenoid concentrations remained stable for weeks, as long as dietary habits did not change. Measurements that had been performed on humans once daily over a period of 1 year demonstrate the effects of stress factors on the carotenoid concentrations in human skin. High levels in dermal carotenoid concentrations correlated well with the intake of food rich in carotenoids by the volunteers, as well as with a reduced number of stress factors that these people were exposed to, as compared to other factors (e.g. smoking, disease). For this reason, the carotenoid concentration in the skin could serve as a suitable marker for enhanced or reduced oxidative processes in the human body.[26]

Measurements in sensitive and robust cattle, as determined by a standardized temperament test, revealed significant differences in the carotenoid concentrations between the two groups. It remains to be proven in studies with a higher number of animals whether individual differences in temperament, expressed in terms of anxiety, result in reduce intradermal β-carotene levels, which might reflect a reduced capacity of such animals to cope with stressful environmental conditions.[65] This is of particular interest to livestock farming, as temperament of cattle has been shown to be related to productivity.[66,67]

Although milk contains only small amounts of the body's carotenoids, the latter contribute to a high extent to the sensory effect of dairy products through their coloring and antioxidant properties.[68] The carotenoid concentration in milk varies depending on breed,[69,70] lactation stage,[71] genetics,[70,71] and nutrition.[68] The question to be answered in the near future will be concerned with how strongly the intradermal β-carotene status and the levels in milk correlate.

Applying MSS for determination of the intradermal carotenoid concentrations in cattle allows noninvasive serial *in vivo* measurements and opens up new perspectives for a better understanding of the carotenoid metabolism and the effects of stress and disease on the oxidative status of cattle. The noninvasive spectroscopic method for measurement of dermal carotenoids promises a versatile application.

5 Conclusion

Carotenoid concentrations were reproducibly measured on bovine udder skin of the same individual animal. The carotenoid concentrations, however, varied significantly between different animals, although all animals had been subjected to the same conditions. Serial measurements on four consecutive days exhibited no significant differences in the intradermal carotenoid concentrations at different time points. Cattle evaluated as robust by a temperament test had significantly higher carotenoid concentrations in their udder skin than animals judged as sensitive ($P < 0.05$).

The present investigations show the feasibility of the determination of carotenoids in bovine skin by applying an optical method based on reflection spectroscopy.

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