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Author response

To the Editor:

In their Letter to the Editor, Hammond and Wooten (2004) offer a critical assessment of the resonance Raman method used to noninvasively measure carotenoid antioxidants in human skin *in vivo*. We are pleased to use this forum to address the issues in the order they were raised.

Raman spectroscopy is a sensitive and highly specific form of vibrational spectroscopy that can be used to identify and quantify chemical compounds. Carotenoid molecules are especially suitable for Raman measurements because they can be excited with light overlapping their visible absorption bands, and under these conditions, they exhibit a very strong resonance Raman response, with an enhancement factor of about 5 orders of magnitude relative to nonresonant Raman spectroscopy (Koyama, 1995). This allows one to detect the characteristic vibrational energy levels of carotenoids through their corresponding spectral fingerprint signature even in complex biologic systems, as in living human tissue. Any off-resonance Raman response from other molecules present in the sampling volume would be strongly suppressed under these conditions and would be buried in background noise.

Approximately 5 y ago, we started to develop resonance Raman spectroscopy for the noninvasive, objective, *in vivo* detection of carotenoid antioxidants in human tissue. Initially we applied the technology to the detection of macular pigments, that is, the carotenoid molecules lutein and zeaxanthin (Bernstein *et al*, 1998, 2002; Gellermann *et al*, 2002a; Zhao *et al*, 2003; Ermakov *et al*, 2004b), which are thought to play a major role in the prevention of age-related macular degeneration. Independent trials using the ocular Raman technology are in progress at several sites worldwide. More recently, we have begun to develop resonance Raman spectroscopy for the detection of carotenoid antioxidants in human skin and mucosal tissue (Hata *et al*, 2000; Ermakov *et al*, 2001, 2003, 2004a; Gellermann *et al*, 2002b, 2003), tissues where various carotenoid species such as lycopene and β-carotene are thought to play an important protective role as well, like in the protection of skin from ultraviolet and short-wavelength visible radiation. Lutein and lycopene may also have protective functions for cardiovascular health, and lycopene may play a role in the prevention of prostate cancer. Skin levels of these species may be correlated with corresponding levels in the internal tissues.

Hammond and Wooten first raise concerns regarding the calibration of the carotenoid Raman signals and point out that to date relatively few quantitative validity results have been published by our group. Certainly, validation is an important aspect. We have shown in our original article (Hata *et al*, 2000) for three excised female abdominal skin tissue samples that carotenoid Raman response and carotenoid concentrations as determined by chemical means (HPLC) do indeed correlate on a rough scale: a sample having a very low concentration as determined by HPLC had a nondetectable carotenoid response, whereas two samples with high and comparable concentrations exhibited high and comparable Raman responses (see Table II in Hata *et al*, 2000). In spite of the fact that skin carotenoid levels are about 2 orders of magnitude lower in skin compared to the human macula, and in view of the fact that their Raman response is superimposed on a large skin autofluorescence background, these first correlation results are very encouraging because they demonstrate that Raman spectroscopy indeed has the potential to identify skin carotenoid levels in a noninvasive optical way. Further data points regarding a direct HPLC/Raman correlation are of course desirable, but they are difficult to perform on a large group of healthy normal subjects in view of the extreme invasiveness of the HPLC method, which typically requires prohibitively large tissue samples. In spite of this obstacle, we have started a study funded by the National Cancer Institute that includes a comparison of carotenoid

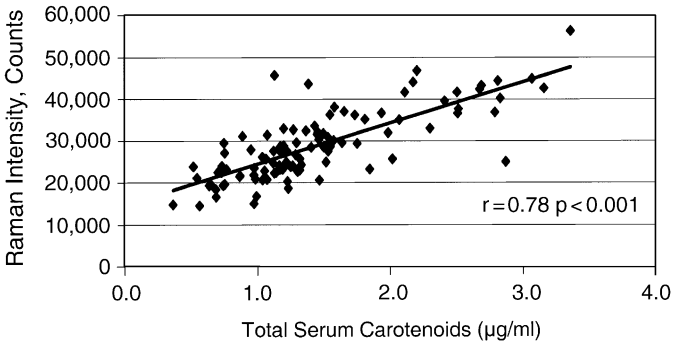

Figure 1
Correlation of skin Raman intensity measured in the inner palm with serum carotenoids determined by HPLC, obtained for a group of 104 healthy male and female adults.

content in living and biopsied skin involving 100 subjects. In this study we will make use of a recently developed, less invasive HPLC protocol carotenoid measurements of small tissue samples (3 × 3-mm punch biopsies; Peng *et al*, 1993).

As indirect supporting evidence regarding validity, we can point to our Raman results obtained with retinal tissue. We observed a near perfect linear correlation over a wide carotenoid range between Raman response and carotenoid content measured by HPLC in human cadaver eyes and in living monkey eye experiments (Bernstein *et al*, 1998; Gellermann *et al*, 2002a). Furthermore, we just finished a study involving a group of 104 human volunteers, where we compared HPLC-derived carotenoid levels of fasting serum with Raman skin levels measured in the inner palm. We obtained a highly significant correlation ($p < 0.001$) with a correlation coefficient of 0.78 (manuscript submitted for publication). The main result of this study is shown in Fig 1. In yet another study, where we measured the Raman response in the palms of 1266 healthy volunteers, we found a wide (27-fold) variation of skin carotenoid level throughout the population, a pronounced positive relationship between self-reported fruit and vegetable intake (a source of carotenoids) and skin Raman response, and 34% reduced skin carotenoid levels in smokers compared to nonsmokers (Gellermann *et al*, 2003). All these results, which are consistent with literature reports for serum data, would be impossible to obtain if the carotenoid Raman detection method were invalid for complex biologic tissue.

Hammond and Wooten then point to the importance of taking into account the "laminar distribution of carotenoids within the skin layers" and speculate that some individuals could have high carotenoid concentrations in the outermost superficial skin layers, whereas other individuals could have similar high concentration levels in the inner layer, and that this would compromise the Raman readings. We agree that this distribution scenario would be a problem: however, to our knowledge there is no evidence for such an effect. To the contrary, superficial dermal layers appear to be a good indicator of carotenoid status in skin, and they have been used successfully by other diagnostic optics tests for this purpose. For example, simple reflection spectrophotometry, which is less specific than Raman spectroscopy and accordingly not as precise, has been successfully used to demonstrate an increase of dermal carotenoid levels with supplementation (Prince and Frisoli, 1993), and it has also been shown that dermal carotenoid levels measured at various sites were highly correlated with serum carotenoid levels (Stahl *et al*, 1998).

Hammond and Wooten next speculate about hemoglobin and melanin as confounding factors in the Raman measurement effects. This is clearly not the case in our measurements for the inner palm, where the laser only penetrates into the bloodless stratum corneum and where melanin is not highly concentrated. In fact, when comparing Raman carotenoid measurements of the inner palm in statistically significant groups of Caucasians and African Americans, we find an insignificant difference in Raman levels. In tissue sites having a thin stratum corneum and/or high melanin content, the mentioned chromophores would of course affect the Raman measurements. Nevertheless, even under these conditions it would be possible to use a suitable correction factor since light propagation in biologic tissue is generally only influenced by two parameters, absorption coefficient and reduced scattering coefficient. Both parameters can be quantified experimentally and used to calculate this correction factor (Pogue and Burke, 1998; Zonios *et al*, 2001).

Hammond and Wooten then turn to an alleged issue of linearity and refer in this context to previous work regarding retinal tissue. Raman spectroscopy, even though it is a linear spectroscopy, scales linearly with concentration only in an optically thin medium. In optically thick media like in geometrically thin but optically dense tissue such as retinal tissue high in carotenoid content, a deviation from linearity would occur, of course. This can be easily taken into account by calibrating the instrument with suitable tissue phantoms, as we already described in detail elsewhere (Gellermann *et al*, 2002a). Regarding skin, we note that the carotenoid concentrations are about 2 orders of magnitude lower than in retinal tissue, where the Raman response was found to scale linearly over a wide and physiologically relevant concentration range. Our recently optimized instrumentation is capable of detecting carotenoid concentrations as low as 1 pg, and it can resolve skin carotenoid spectra with a signal-to-noise ratio as high as 20:1. It is capable of detecting small changes in carotenoid content, as caused by dietary supplementation (Gellermann *et al*, 2003; Ermakov *et al*, 2004a). Most importantly, a clear correlation between Raman response and carotenoid content can be established as a consequence of these properties (see Fig 1).

Hammond and Wooten then invoke changes in epidermal morphology as potential confounding factors. We disagree in view of our results and those in the literature that state that stratum corneum is generally uniform in the normal state across individuals (Shaw, 1991). Even in diseased skin, correction factors based on experimentally obtainable absorption and reduced scattering coefficients (see above) may not be needed in some cases. For example, squamous cell carcinoma tissue sites, as investigated in Hata *et al* (2000), appeared optically quite similar upon visual inspection to adjacent healthy tissue sites.

Finally, Hammond and Wooten briefly question the specificity of the techniques regarding selective detection of carotenoids. It is clear that all resonantly excited

carotenoid species contribute to a Raman response. In our current Raman method, which uses blue and green laser lines, we capture the most prevalent carotenoids in skin, that is, lycopene, β-carotene, lutein, and all their isomers, because all absorb in the same spectral vicinity. As described in Ermakov *et al* (2003) and Ermakov *et al* (2004a), it is possible to selectively determine lycopene as well as the composite concentration of the remaining carotenoids, revealing significant individual differences in the relative dermal beta carotene/lycopene compositions.

In summary we strongly disagree with Hammond and Wooten's assertion that resonance Raman spectroscopy is unsuitable for quantitative detection of carotenoids in skin. It is highly specific, sensitive, and precise, and it allows one to rapidly assess dermal carotenoid content in large populations with excellent correlation to serum levels. To our knowledge there are no serious confounding factors for the technology, and it has exciting application potential. In the nutritional supplement industry it is already being used as an objective, portable device for the monitoring of effect of carotenoid-containing supplements on skin tissue carotenoid levels. In cancer epidemiology, it may serve as a noninvasive novel biomarker for fruit and vegetable intake, replacing costly plasma carotenoid measurements with inexpensive and rapid skin Raman measurements. Finally, owing to its capability of selectively detecting lycopene, the technology may be useful to investigate a specific role of lycopene in the prevention of prostate cancer and other diseases.

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