

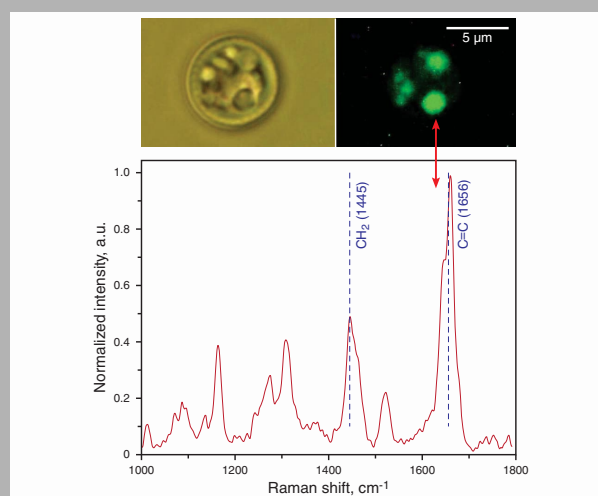
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Abstract: Raman spectroscopy offers a powerful alternative analytical method for the detection and identification of lipids/oil in biological samples, such as algae and fish. Recent research in the authors' groups, and experimental data only very recently published by us and a few other groups suggest that Raman spectroscopy can be exploited in instances where fast and accurate determination of the iodine value (associated with the degree of lipid unsaturation) is required. Here the current status of Raman spectroscopy applications on algae is reviewed, and particular attention is given to the efforts of identifying and selecting oil-rich algal strains for the potential mass production of commercial biofuels and for utilization in the food industry.



Characterization of algae cells: differential interference contrast (DIC) image (left), fluorescence image (right), and Raman spectrum of a lipid body within the algae

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Characterization of oil-producing microalgae using Raman spectroscopy

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1. Introduction

It is widely believed that one of the promising alternatives for satisfying the increasing demands of the human population for energy sources is the production of carbon-based fuels from plants. Through the process of photosynthesis, plants convert the energy of solar radiation into chemical energy, stored in the molecular building blocks of life – proteins, lipids, carbohydrates, etc. These provide the energy for most life forms on Earth. There are several basic strategies for producing fuel from plant biomass; the clas-

sical approach utilizes highly-developed plants including agricultural crops. However, this solution competes with food production, and thus requires vast, valuable land and water resources, and one sees this competition already reflected in rapidly rising commodity prices.

More recently, attention has turned to the potential exploitation of oxygenic, photo-autotrophic microorganisms (algae) for the production of higher-generation biofuels [1–12]. This alternative approach, while still very much in its infancy, is gaining credibility: large, estab-

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lished multi-national corporations have announced major research projects to embark on proof-of-principle or large-scale testing of algae cultivation for mass generation of biofuels. Amongst others, the main players currently are (i) the ExxonMobil Corporation, in collaboration with Synthetic Genomics; (ii) BP Amoco plc, in cooperation with Martek Biosciences Corporation; and (iii) most recently, in the UK, the Carbon Trust announced the “Algae Biofuel Challenge”, consisting of twelve coordinated projects, mainly from leading UK universities.

As in the production of biofuel from agro-biomass there is a potential caveat in the use of algae. In marine and freshwater systems, thousands of organisms interact with a highly dynamic aquatic environment. Phytoplankton/algae, serve as the base of the aquatic food chain, because of the ability to synthesize poly-unsaturated fatty acids. Phytoplankton production is mostly consumed by zooplankton which is, subsequently, consumed by fish. The last members of this overall food chain are humans when they eat fish. It is quite obvious that in coastal areas, humans eat abundant supplies of fish species that contain poly-unsaturated fatty acids (PUFA) and highly unsaturated fatty acids (HUFA). HUFA is a subset of PUFA with ≥ 20 carbon atoms and ≥ 3 carbon double-bonds (C=C). An example of a HUFA-group molecule, used in our study, is Omega-3 fatty acid, EPA (eicosapentaenoic acid; 20:5, with 20 carbon atoms and 5 carbon double-bonds) [13]. The choice of food consumed by humans does not always satisfy nutritional requirements for both n-3 (Omega-3) and n-6 (Omega-6) fatty acids. Because choosing a particular type of food is mainly based on proximity, convenience, taste/odor and digestibility, rather than on specific nutritional requirements [13], by not rating fatty acids adequately people living in inland areas, where it is difficult and expensive to eat fresh seawater fish, might be adversely affected by this choice. In this context it should be noted that algal supplements of Omega-3 fatty acids (or different algal oil mixtures) have recently appeared on the market.

In the light of these two challenges related to biotechnological exploitation of algae and living microorganisms, a reliable analytical method is needed, which can estimate whether useful fatty acids are present in certain foods, or where in the food chain they are transferred; and equally it is desirable to identify and select specific algae for convenient, efficient growth to produce economically viable biofuel. In particular, such a (fast) method for the identification and analysis of lipids/oil would need to be able to estimate their so-called iodine value, or IV.

Note that the iodine value reflects the degree of unsaturation of a fatty acid (or oil), and describes its particular structural indices (together with the saponification value, and the hydroxyl value [14]). As such, IV is an important parameter, and it constitutes part of the current biofuel and food industry standards. In some European standards, the IV has been included, usually being set to a maximum of 115 [14]. Traditionally, IV determination is by chemical analysis related to carbon double-bond reactiv-

ity [14–18]; its value is derived from the technique which adds iodine to unsaturated oils/fats to react quantitatively with the carbon double-bonds [16]. The addition of iodine to the unsaturated fatty acid chains of the oil molecule is via reaction/binding with the carbon double-bonds. Thus, the IV of oils measures the amount of C=C present in a sample; it is expressed as grams of iodine per 100 g of sample. However, this analytical technique involves several time-consuming steps, as is well described in [14–18], so that complete analysis takes about 30–45 minutes [16]. Thus, a simpler and speedier analytical technique would be rather desirable, with potential benefits in food industry control-process applications, and in biofuel applications (for example, to estimate a fuel index [14]).

It is this particular challenge which is leading to the development of a methodology, based on Raman spectroscopy for fast and accurate IV estimation: it is calculated from intensity ratios of specifically selected Raman bands. This technique promises to enable rapid screening for optimal algae selection for biofuel applications on-line within the bioreactor, as well as to contribute to the understanding of lipid profiles in fish species *in-situ* in the aquaculture industry, where fish have access to an abundant plankton/algae population containing different oil mixtures. The authors' groups, and others, are currently working toward proof-of-principle solutions.

2. Raman spectroscopy for algal investigation – current status

In order to utilize algae for efficient lipid/oil production (algae synthesize fatty acids in lipid bodies), the optimal cultivation parameters have to be determined, which – in turn – lead to high production of oil-precursor molecules in the selected cell line. Therefore, techniques are required allowing for rapid characterization/identification of algae species, and specifically to determine the degree of unsaturation of constituent fatty acids in algal lipid bodies. Raman spectroscopy can provide this biochemical information and reveal the molecular composition of the studied microorganisms and biological samples for a vast variety of applications [19–36]. The detection and identification of important, basic molecules in biological samples by using Raman spectroscopy has been summarized in two relatively recent, generic reviews [37,38].

Those who have applied Raman spectroscopy to the analysis of photo-synthesizing organisms, such as algae, are all too aware of the fact that their spectroscopy is complicated by the underlying strong fluorescence of omnipresent pigments that might obscure the characteristic Raman spectral features. This poses a severe challenge, and hitherto the use of Raman spectroscopy has been restricted to relatively few algal species. Consequently, the number of publications recently covering the topic and, also, the number of research groups involved in this field of research is still rather small, although visibly increasing in

Algae species	Reference	Estimate of the degree of unsaturation/iodine-value of algal oil
<i>Dunaliella tertiolecta</i>	[39,40]	no
<i>Chlorella sorokiniana</i> <i>Neochloris oleoabundans</i>	[41]	no
<i>Botryococcus braunii</i>	[42]	no
<i>Botryococcus braunii</i> <i>Neochloris oleoabundans</i> <i>Chlamydomonas reinhardtii</i>	[43]	yes
<i>Trachydiscus minutus</i> <i>Botryococcus sudeticus</i> <i>Chlamydomonas sp.</i>	[44]	yes

Table 1 Algae species investigated in Raman spectroscopy research

the last year. Because of being in an early stage of development these publications on Raman spectroscopy of algae are scattered over a range of journals (for example, Web of Science tracks altogether six journals on this topic [39–44]), with the majority of work published during 2010 and 2011. The algal species with recognized prospects for biofuel production which were investigated by five research groups worldwide are summarized in the Table 1. The most widely studied species is *Botryococcus*, which was examined in detail by three research groups; the species with the highest IV value was found to be *Trachydiscus*. Thus far only two groups have been involved in systematic research on estimating the unsaturation degree/iodine value within algal samples (see Table 1).

The last four years have seen an increase in interest in applying Raman spectroscopy to the study of algae. The work performed in 2007 in Beardall's laboratories at Monash University (Australia) [39,40] may be seen as pioneering; it focused on *in vivo* Raman spectroscopy for predicting the nutrient status of individual algal cells. The researchers found that when using 780 nm laser excitation the associated spectra of cells revealed a range of Raman bands mainly attributed to chlorophyll and carotene. In-depth research in this direction demonstrated that the relative intensities of chlorophyll and carotene bands changed under nitrogen limitation during the cultivation of the algae. Thus, multivariate classification methods could accurately predict the nutrient status of the cells, based on the analysis of the Raman spectral data. In this early work, however, the authors did not identify Raman transitions which can be attributed to lipids/fatty acids, so that no data on unsaturation of algae lipids were included.

Preliminary feasibility studies on using Raman spectroscopy of algae were reported in early 2010 [41]. These were performed on two algal species, namely *Chlorella sorokiniana* and *Neochloris oleoabundans*, which are po-

tential candidates for biofuel production. Raman signals due to storage lipid (specifically triglycerides) were identified in nitrogen-starved *C. sorokiniana* and *N. oleoabundans* samples. Also, the authors report that the observed fluorescence background, while acquiring Raman signals from the algae, exhibited a time-dependence, which could be characterized by general power-law decay, interrupted by sudden high-intensity fluorescence events. They concluded that the decreasing trend was likely to be the result of photo-bleaching of cell pigments due to prolonged intense laser light exposure; but the occurrence of the sudden high-intensity fluorescence bursts eluded full understanding. But as in the work of the Beardall group, outlined in the previous paragraph, the authors did not extract the relationship between the lipid (or triglyceride) concentration in algae and the associated Raman signal intensity, in order to establish a standardized lipid quantification method. Thus, data on unsaturation of algae lipids are not given either.

Raman spectroscopy on *Botryococcus braunii* algae was reported in mid-2010 [42]. In said work the authors focused solely on mapping the presence and location of methylated botryococcenes within a colony. Density function theory calculations were used to determine the Raman spectra of all botryococcenes to compare computed theoretical values with those observed. In the study specific Raman spectroscopic characteristics for botryococcenes of *Botryococcus braunii* have been identified. Once again, no data values for unsaturation of algae lipids were published.

Quite recently *in vivo* lipid profiling of oil-producing algae has been described, using single-cell laser-trapping Raman spectroscopy [43]. In this study it was demonstrated that quantitative determination of the degree of unsaturation and transition temperatures of constituent lipids within algae could be achieved reliably. Single living algal cells were held in place within an optical trap while collecting Raman spectra. The authors specifically highlighted the analytical capability of their approach, and they demonstrated that their quantification algorithms were applicable to a range of organisms in the field of lipidomics.

Finally, the groups of the authors of this review recently demonstrated spatially resolved Raman spectroscopy to determine the effective iodine value in lipid storage bodies of individual algal cells [44]. The Raman spectra were collected from different algal species immobilized in an agarose gel, thus preventing them from moving out of the tightly focused region of the probe laser beam in order to maintain high spatial resolution within lipid bodies. We exploited that the principal parameter characterizing the algal lipid is the degree of unsaturation of the constituent fatty acids can be quantified by the iodine value. Crucially, in our work the IV is conveniently estimated from information contained within the Raman spectra, without the need to add any chemicals to the cells. To be precise, we used the characteristic peaks in the Raman spectra at 1656 cm^{-1} (*cis*-C=C stretching mode) and 1445 cm^{-1} (CH₂ scissoring mode) as the markers defining the ratio of unsaturated-to-saturated carbon-carbon bonds

of the fatty acids in the algal lipids. For quantitative IV-determination a calibration curve was generated based on pure fatty acids of known iodine value. This calibration was subsequently applied in the IV estimate for lipids stored in algal cells. It could be unequivocally shown that the iodine value differed significantly for the various algal species. These estimates based on Raman spectroscopy were validated using the established technique of gas chromatography and mass spectroscopy (GC-MS); indeed, excellent agreement was found.

In order to further underpin the validity and the potential strength of the Raman spectroscopic techniques towards estimating the iodine value of algae, and other species, accurately and rapidly, in the following section we present some recent results from our groups, as yet unpublished.

3. Characterization of lipids using Raman spectroscopy

3.1. Raman micro-spectroscopy

The Raman response of any sample to laser excitation can be described by a relatively simple relation, which relates the Raman light intensity to the exciting-laser light intensity and the number of interacting molecules, and is given by

$$I_{Raman}(\nu) = a(\nu)S_{if}(\nu)NI_{laser}, \quad (1)$$

where the Raman light intensity $I_{Raman}(\nu)$ is a function of frequency (associated with the vibrational transitions modes); $a(\nu)$ is a proportionality function, which includes geometrical factors in the light collection system and spectral sensitivities of the detection system; $S_{if}(\nu)$ represents the Raman transition probability from an initial level i to a final level f ; N is the number of molecules contributing to a particular vibration mode; and I_{laser} is the intensity of the laser initiating the Raman transitions. Relation (1) means that, if no experimental parameters change, the observed spectral peaks are directly proportional to the number of Raman scattering molecules. However, while the (quantum) transition probabilities are intrinsic, non-varying quantities, the behavior of the proportionality function $a(\nu)$ is difficult to ascertain quantitatively. Specifically, the spectral response functions of a detection system (comprising normally a spectrometer and a CCD array detector) and of the various filters in the light collection path are not easy to characterize exactly. But as long as one only is interested in the relative ratio between spectral features – as is the case in the methodology described here – this is not a problem, as long as the experimental conditions are kept unchanged.

In the context of the experiments which are subject of this review, not only does one aim at the general spectroscopy of an algae sample but the interest is in the identification of a particular lipid, ideally with spatial resolution. As was mentioned in Sec. 2 it now has become possible to map the storage lipid bodies of individual algae

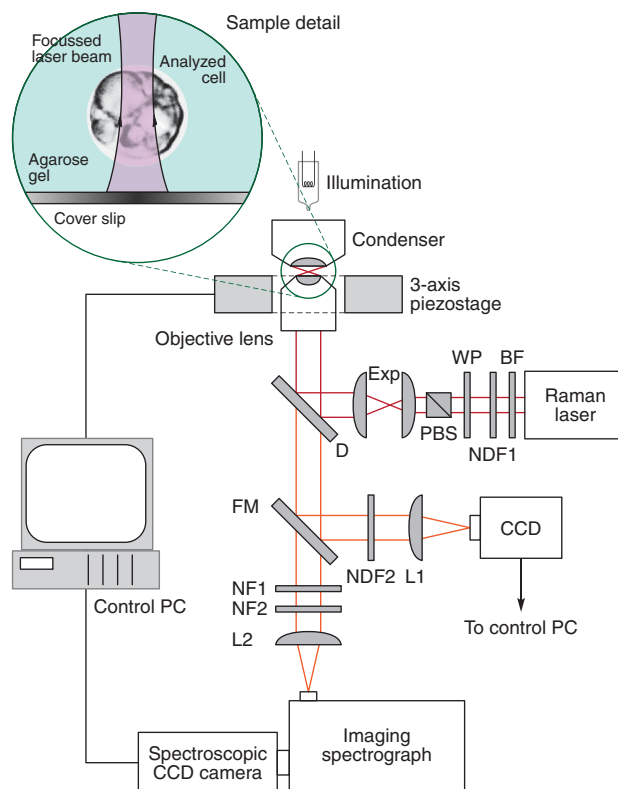


Figure 1 (online color at www.lphys.org) Schematic diagram of the experimental setup for Raman micro-spectroscopy of single algae. BF – band pass filter, D – dichroic mirror, Exp – beam expander, FM – flipping mirror, L1, L2 – lenses, NDF1, NDF2 – neutral density filters, NF1, NF2 – notch filters, PBS – polarizing beam splitter, and WP – lambda-half wave plate. The inset shows the detail of the immobilized algal sample within the laser focus

using Raman micro-spectroscopy; this was demonstrated for three specific species, namely *Botryococcus sudeticus*, *Chlamydomonas sp.*, and *Trachydiscus minutus* [44], but one should be able to extend the approach to other species with ease. The intensities of the Raman spectral peaks that correspond to the saturated and unsaturated carbon-carbon bonds in lipid molecules are used to estimate the degree of unsaturation in the lipid bodies, in a similar fashion to previously published work on margarines, seeds and vegetable oils [45–48]. Representative for the various possible experimental implementations we briefly describe our own apparatus.

3.1.1. Experimental

Raman micro-spectroscopic experiments with living algal cells were carried out using an in-house built experimental system, incorporating a custom-designed inverted microscope frame [44]. The conceptual layout of this system is shown in Fig. 1.

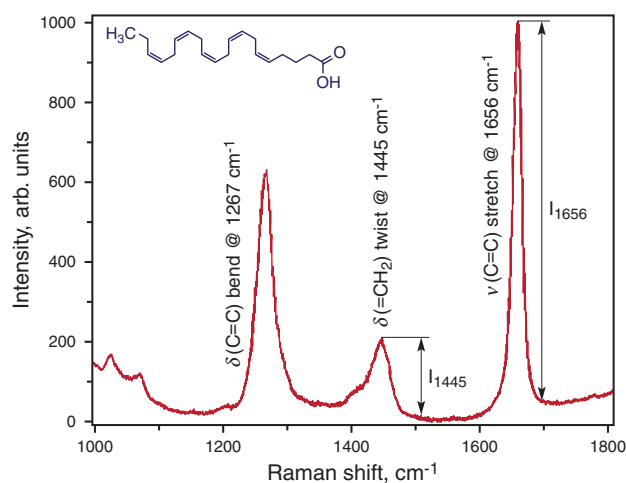


Figure 2 (online color at www.lphys.org) Raman spectrum of EPA (Eicosapentaenoic acid – Omega-3 fatty acid) used for constructing calibration curve for IV estimation

Briefly, the Raman laser excitation beam (from a Coherent 899-01 Ti:Sapphire laser, operating at $\lambda = 785$ nm, with beam diameter $d_{beam} = 0.6$ mm) was delivered to the setup via an optical fiber. The power of the Raman excitation laser could be adjusted gradually by the neutral density filter NDF1 with continuously variable optical density (Thorlabs). The maximum laser power available for excitation was measured to be approximately 60 mW at the location of the specimen. The laser beam was focused onto the specimen with an IR-optimized water-immersion objective lens (Olympus UPLSAPO 60 \times , NA = 1.2). The Raman scattered light was collected through the same objective lens, dispersed with a 600 grooves/mm diffraction grating, imaged onto the chip of a high-sensitivity, liquid-nitrogen-cooled spectroscopic CCD camera (Princeton Instruments Spec-10:100BR/LN), and recorded using the camera's control software (Princeton Instruments WinSpecTM). Post-acquisition processing of the spectra was done off-line, using custom-written routines incorporated into MathWorks's MatlabTM software.

The setup depicted in Fig. 1 was utilized to measure the degree of unsaturation in single, immobilized, living algal cells and constituted a combination of an optical trap and a micro-Raman system. Spectrum acquisition times to collect spectra from a single micro-algal cell were typically < 10 s [44].

3.1.2. Spectrum processing and analysis

Despite using a near-infrared laser for Raman scattering excitation, and focusing the laser beam directly into the lipid storage bodies, the collected Raman spectra still display a noticeable non-specific fluorescence background. In order to extract quantitative information from the experi-

Fatty acid	IV value
Palmitic acid	0
Oleic acid	90
Mixture-1 (oleic acid/arachidonic acid; ratio 10/5 mg)	158
Linoleic acid	180
Mixture-2 (oleic acid/arachidonic acid; ratio 6/5 mg)	191
Arachidonic acid	330
Eicosapentaenoic acid (EPA)	420

Table 2 Calibration samples and their IV

mentally obtained spectral data, we adopted the rolling circle filter (RCF) technique for background removal [44,49]. In principle, the RCF procedure constitutes a high-pass signal filter that allows separating the narrow Raman spectral peaks from the background; note that the filter radius of curvature has to be significantly larger than the probed line profile. With an appropriate choice of the filter parameters (filter width and number of filter passes), background can be effectively removed without causing any significant distortion of the signal peaks. After background removal, the actual analysis of the Raman spectra can be carried out.

Fig. 2 shows a typical Raman spectrum, here for a small oil droplet (pure EPA was used for calibration). It was administered directly on top of a cover slip, and the Raman excitation beam was focused inside the droplet, approximately 20 μm away from the liquid-glass interface; this mimics the configuration as later encountered for the algae samples.

3.1.3. Determination of iodine values

In our experiments, we determined the ratio of unsaturated-to-saturated carbon-carbon bonds in algal lipid molecules. For this two characteristic spectral peaks were used: the first at 1656 cm^{-1} corresponds to the *cis*-C=C stretching mode proportional to the amount of unsaturated C=C bonds; and the second at 1445 cm^{-1} is associated with the CH₂ scissoring mode, whose intensity is proportional to the amount of saturated C-C bonds. Both peaks are highlighted in Fig. 2.

From the ratio of intensities I_{1656}/I_{1445} , the average ratio of double-to-single carbon-carbon bonds $n(\text{C}=\text{C})/n(\text{CH}_2)$ in the specimen – associated with specimen mass unsaturation – can be estimated [44]. This is accomplished by constructing a calibration curve that relates I_{1656}/I_{1445} to $n(\text{C}=\text{C})/n(\text{CH}_2)$ for pure fatty acids of varied degree of the hydrocarbon chain unsaturation (see [44]). On the basis of the published iodine values for the pure fatty acids used in our calibration, it is possible to directly convert the measured values of $n(\text{C}=\text{C})/n(\text{CH}_2)$ to the iodine values for a given sample.

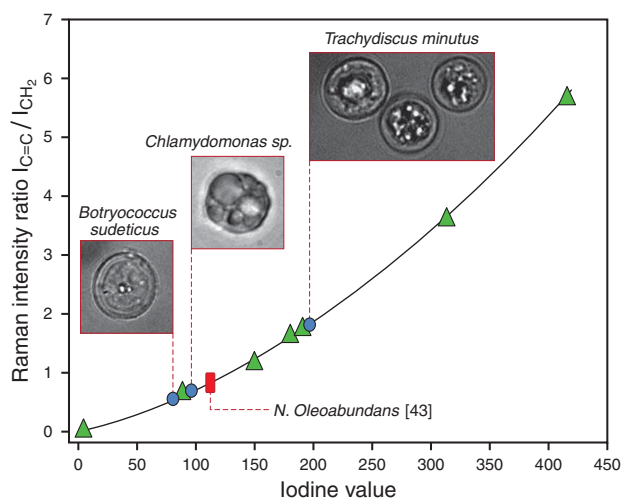


Figure 3 (online color at www.lphys.org) Calibration curve for estimating the iodine value (IV) from the Raman spectral data. The continuous line is a parabolic fit of the experimental data (see text). Error bars are within the size of the data symbols

All in all, seven reference samples were utilized in the calibration, representing pure fatty acids and two oil mixtures of varied degree of unsaturation [44]; these are collated in Table 2. The IV range of 0–420 covers virtually all biologically relevant fatty acids (with the highest value for EPA, IV = 420).

The resulting Raman calibration data are shown in Fig. 3, indicated by the (green) triangular data points. The data can be fitted well using a parabolic function which represents the measured calibration points in the form

$$y = 2.0 \times 10^{-5}x^2 + 5.3 \times 10^{-3}x + 1.86 \times 10^{-2},$$

where y is the Raman intensity ratio I_{1656}/I_{1445} and the variable x stands for the iodine value. The fit is rather good with $R^2 = 0.9974$.

Recall that the only other group thus far, apart from our own, which has addressed estimating the degree of unsaturation directly from Raman spectra is that of H.W. Wu and co-workers; in their recent publication they provide some intensity ratios for C=C/CH₂ band contributions [43]. We have included H.W. Wu's Raman peak ratio result for the algal species *Neochloris oleoabundans* – which they give as about 0.9 – in Fig. 3 as well. According to the calibration plot, one deduces that its algal lipid content is close to IV \cong 115. This value is higher than that for the two algal species (*Botryococcus sudeticus* and *Chlamydomonas sp.*), which were investigated in full detail at Brno, but lower in comparison to *Trachydiscus minutus*.

As was mentioned in the introduction, one of the last members in the food chain, which starts with algae, are fish. It is therefore obvious that fish species being high in poly-unsaturated fatty acids (Omega-3 and Omega-6), reflect the amount and composition of these fatty acids of

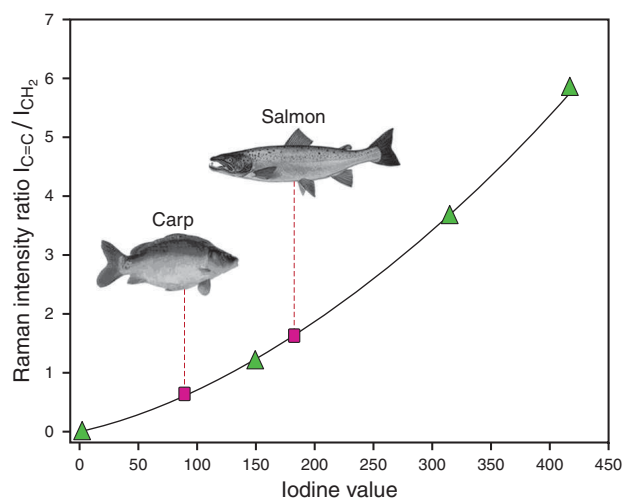


Figure 4 (online color at www.lphys.org) Calibration curve for estimating the iodine value (IV) from the Raman spectral data for two fish species – carp and salmon. The calibration reference data are the same as in Fig. 3

the plankton/algae they feed on. Thus, the method of iodine value determination described here may also be useful for characterizing fish oils. Results of IV estimates for two fish species are shown in Fig. 4, using the same calibration curve as that of Fig. 3.

From the figure one extracts that Salmon has an IV \cong 180, which implies high amounts of Omega-3 fatty acids (EPA and DHA) in its fish oil. Carp as a freshwater fish (domesticated and reared as food fish in the Czech Republic) has an IV \cong 90, which means a lesser amount of Omega-3 fatty acids; still, carp may be seen – from a nutritional point of view – as being an excellent choice for the uptake of beneficial fatty acids.

Finally, in order to estimate the average number of C=C bonds within particular algal (or fish) oil, in Fig. 5 the Raman peak ratios I_{1656}/I_{1445} of the pure fatty acids were plotted against the number of C=C bonds listed in Table 3.

As in the IV-calibration, the data fit extremely well ($R^2 = 0.9992$) to a parabolic function in the form $y = 0.1260x^2 + 0.5237x + 0.0113$, where y is the Raman intensity ratio I_{1656}/I_{1445} and the variable x stands for the number of C=C bonds. Inserting the Raman peak ratio data for the algae, and fish oils, the results suggest that the lipid mix of the included species exhibit average double bonds per lipid molecule-equivalent of about 1.0 to 2.5.

3.2. Verification of Raman spectroscopic data by GC-MS

Whenever a new technique is to be established as a reliable analytic method, one is well advised to verify results by

Fatty acid	Content w/w, %	Molecular weight	MFE	n(C=C)	n(CH ₂)	Contribution (C=C)	Contribution (CH ₂)
Mystic acid	5.13	228.37	0.0225	0	12	0	0.2696
Palmitoleic acid	7.48	254.41	0.0294	1	12	0.0294	0.3528
Palmitic acid	9.90	256.42	0.0386	0	14	0	0.5405
Linoleic acid	12.95	280.45	0.0462	2	12	0.0924	0.5541
Oleic acid	9.12	282.46	0.0323	1	14	0.0323	0.4520
Stearic acid	1.99	284.48	0.0070	0	16	0	0.1119
Arachidonic acid	8.54	304.50	0.0280	4	10	0.1122	0.2805
EPA	30.25	302.45	0.1000	5	8	0.5001	0.8001
Behenic acid	11.58	340.58	0.0340	0	20	0	0.6800
Ligoneric acid	3.06	368.63	0.0083	0	22	0	0.1826
Sum	100.00		0.3463			0.7663	4.2242

Table 3 Fatty acid composition of *Trachydiscus minutus* from GC-MS analysis [44]. Ratios of the sum of C=C contribution to the sum of CH₂ contribution were used for IV-estimation. Annotation: MFE – molar fraction in the extract, n(C=C) – number of double bonds per molecule, and n(CH₂) – number of CH₂ groups per molecule

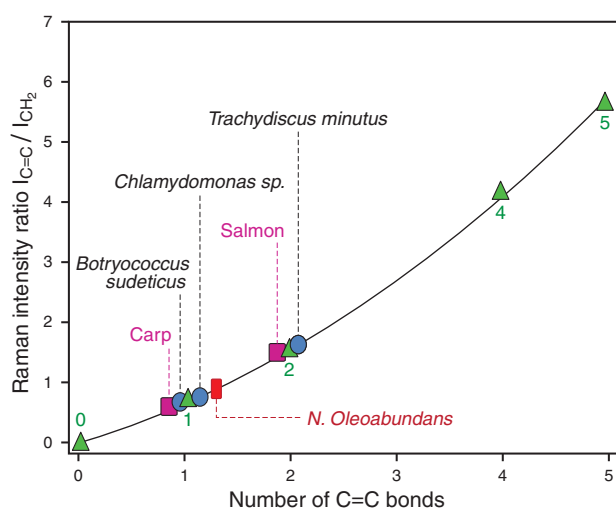


Figure 5 (online color at www.lphys.org) Dependence of the observed Raman intensity ratios of the Raman peaks at 1656 and 1445 cm⁻¹ on the number of double bonds C=C. For the pure fatty acid samples one encounters integer numbers, for the algae and fish oil samples with lipid mixtures one finds fractional, average number of C=C bonds. The labels 0, 1, 2, 4, and 5 indicate the calibration samples palmitic, oleic, linoleic and arachidonic acids, and EPA, respectively. Error bars are within the size of the data symbols

(at least) one other method. We verified the data obtained from Raman micro-spectroscopic measurements by comparing them with GC-MS analysis of the algal fatty acid composition of the various algae species. Exemplary, this approach is described for *Trachydiscus minutus*, which is an excellent source of EPA.

To calculate the mean IV of the algal fatty acid mixtures from the GC-MS results, we determined the average mass unsaturation ratio $N_{C=C}/N_{CH_2}$ from the data presented in Table 3, and subsequently, we employed the calibration presented in Fig. 4 of [44]. From the table data this unsaturation ratio is 0.181, yielding – according to the calibration in [44] – a theoretical Raman peak ratio of 1.73 ± 0.08 ; this is in nearly perfect agreement with the measured Raman peak ratio of $\cong 1.78 \pm 0.05$, which translates into an IV $\cong 195$. This cross-referencing result confirms that Raman spectroscopy may indeed be seen as a reliable, versatile and non-invasive analysis method in applications in lipid-engineering and industrial processing, where real-time knowledge of the lipid content is essential. Fast determination of the IV could potentially reduce time and the effort of identification and selection of the best possible algal strains (identification of oil within algal lipid bodies) for the mass production of commercial biofuel and for food industry applications, including fish feeds in a pond-production system.

Note that we could also indirectly compare our data for estimating the IV for carp from Raman spectroscopy to GC-MS results. J. Mráz and J. Pickova list the fatty acid composition of carp samples in full detail [50]. For this, we estimated the IV from GC-MS data (as for the *Trachydiscus minutus* case above, and using the data from Table 2 in [50] we arrive at IV $\cong 80$ for carp. This is in rather good agreement with our Raman data evaluation of IV $\cong 90$, considering the completely different origin of the samples used in the analysis.

4. Conclusions and future outlook

We have attempted to review recent applications of Raman spectroscopy to algal research, and in particular its use for

the determination of important parameters of fatty acids, such as iodine value estimation and the average number of carbon-carbon double-bonds.

Raman spectroscopy can be combined with optical tweezers and with micro-fluidic chips, so that such a hybrid-instrument should make it possible to measure nutrient dynamics and metabolism *in vivo*, in real-time, and label-free. Thus, with this type of bio-sensing one may easily detect population variability and, consequently, changes in iodine value. It is now quite feasible to employ Raman spectroscopy-based sensors to sort cells according to the degree of unsaturation (IV) in lipid storage bodies of algal cells in-line and in real-time. We have also shown that various algal species display significantly different iodine values, which has important implications for biotechnological exploitation of particular algae.

Our spectroscopic data agree well with the results obtained in cross-calibration measurements based on gas chromatography, so that Raman spectroscopic methodologies might soon be considered as an experimental standard for characterizing the fatty-acid composition of microorganisms. We believe that Raman spectroscopy – being a reliable, label-free and non-invasive technique – will be of significant assistance to research groups currently being involved in, or intending to join, the quest for sustainable biofuel generation and algal food applications, ranging from microbiology and biotechnology, over systems biology and genetic engineering, to pharmaceutical and food industry uses.

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