VIBRATIONAL SPECTROSCOPY (FTIR-ATR AND FT-RAMAN)
A Rapid and Useful Tool for Phycocolloid Analysis

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Abstract: The wide industrial application of phycocolloids (e.g. alginates, agar and carrageenans) is based on their particular properties to form gels in aqueous solution. Recently, new spectroscopic techniques have provided more accurate identification of the natural composition of the polysaccharides produced by these seaweeds. With the combination of two spectroscopic techniques (FTIR-ATR and FT-Raman) it is possible to identify the principal seaweed colloids in ground seaweed samples as in extracted material. Since the seaweed samples receive the minimum of handling and treatment (e.g. they are simply dried and ground), the composition determined represents, as accurately as possible, the native composition of the phycocolloids.

1 INTRODUCTION

Many seaweeds produce hydrocolloids, associated with the cell wall and intercellular spaces. Members of the red algae (Rhodophyta) produce galactans (e.g. carrageenans and agars) and the brown algae (Phaeophyceae) produce uronates (alginates). Carrageenans represent one of the major texturising ingredients used by the food industry; they are natural ingredients, which have been used for decades in food, excepients applications and are generally regarded as safe (GRAS).

The phycocolloid *carrageenin*, as it was first called, was discovered by the British pharmacist Stanford in 1862 who extracted it from Irish moss (*Chondrus crispus*). The name was later changed to carrageenan so as to comply with the ‘-an' suffix for the names of polysaccharides. The modern carrageenan industry dates from the 1940s, receiving its impetus from the dairy industry where carrageenan was found to be the ideal stabilizer for the suspension of cocoa in milk chocolate.

The commercial carrageenans are normally divided into three main types: kappa, iota and lambda-carrageenan. Generally, seaweeds do not produce these idealized and pure carrageenans, but more likely a range of hybrid structures. Several other carrageenan repeating units exist: e.g. xi, theta,

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beta, mu and nu. The precursors (mu and nu), when exposed to alkali conditions, are modified into kappa and iota, respectively, through formation of the 3,6-anhydro-galactose bridge (Rudolph, 2000).

Infrared (IR) spectroscopy was, until recently the most frequently used vibrational technique for the study of the chemical composition of phycocolloids. This technique presents two main advantages: it requires minute amounts of sample (milligrams), and it is non-aggressive method with reliable accuracy (Givernaud-Mouradi, 1992; Pereira *et al.*,2003). However, conventional IR spectroscopy requires laborious procedures to obtain spectra with a good signal/noise ratio (Chopin and Whalen, 1993). This limitation was overcome with the development of interferometric IR techniques (associated with the Fourier transform algorithm), known as FTIR spectroscopy (Fourier Transform IR). More recently, Pereira and collaborators had used a technique of analysis on the basis of FTIR-ATR (from Attenuated Total Reflectance) spectroscopy, allowing for the determination of the composition of the different phycocolloids from dried ground seaweed, without having to prepare tablets of KBr (Pereira, 2006; Pereira and Mesquita, 2004).

In contrast to FTIR, the application of conventional Raman spectroscopy was limited until recently, due to need for an incident visible laser in dispersive spectrometers: the visible laser light often excites electronic transitions in biochemical samples, which can lead to either sample degradation or strong background signal from unwanted laser-induced fluorescence. The use of Nd:YAG lasers operating at 1064 nm (far from the visible region) in interferometric spectrometers has been generalized to decrease the fluorescence level and avoid sample degradation. The modern FT-Raman spectrometers have been used to produce good quality Raman spectra from seaweed samples. (Matsuhiro, 1996; Pereira *et al.*, 2003).

In this work, a combined FTIR-ATR and FT-Raman spectroscopy study were used to identify the colloid produced by one of the principal source of carrageenans, the red algae *Chondrus crispus*. Since the analysis of ground seaweed samples required minimal treatment (the seaweeds are simply dried and ground), the determined composition represents, as accurately as possible, the natural colloid composition.

2 MATERIALS AND METHODS

2.1 Algal Material and Standard Samples of Phycocolloids

Specimens of red algae (Rhodophyceae) *Chondrus crispus* are collected in the central zone of the western coast of Portugal (wild specimens) and other are cultivated in Canada (lambda strain). Standard samples were obtained from Sigma (type IV, C-3889) and CP Kelco (pure lambda-carrageenan).

The sample composition and purity were controlled by NMR.

2.2 Preparation of Ground Seaweed Samples for FTIR-ATR and FT-Raman

The seaweed samples were rinsed in distilled freshwater to eliminate salt and debris from the thallus surface and dried to constant weight at 60 ºC. The dried seaweeds were finely ground in order to render the samples uniform. For FTIR analysis the samples do not need additional treatment. The analysis by FT-Raman requires that these are without pigmentation. The lack of pigmentation can be achieved by sun drying (process used by collectors/producers of commercial seaweeds) or by pigment elimination in the laboratory by the addition of acetone/methanol moisture (V/V) or by the addition of calcium hypochlorite solution (4%, 30/60 s, 4 ºC) (Pereira, 2004).

2.3 Phycocolloid Extraction

Before phycocolloid extraction, the ground dry material was rehydrated and pre-treated in acetone followed by ethanol to eliminate the organosoluble fraction (Zinoun and Cosson, 1996).

For extraction of the native phycocolloid, the seaweed samples were placed in distilled water (50 ml/g), pH 7 at 85º C for 3 h. For an alkaline-extraction (resembling the industrial method), the samples were placed in a solution (150 mL/g) of NaOH (1 M) at 80-85 ºC for 3-4 h according to Pereira and Mesquita (2004), and neutralised to pH 6-8 with HCl (0.3 M).

The solutions were hot filtered, twice, under vacuum, through cloth and glass fibre filter. The extract was evaporated under vacuum to one-third of the initial volume. The carrageenan was precipitated by adding the warm solution to twice its volume of ethanol (96 %).

Table 1: Carrageenan composition determined by vibrational spectroscopy (FTIR-ATR and FT-Raman) and NMR.

Species/Sample	Lifecycle phase	Origin	Carrageenan		
			Yield[1]	Alkali extracted[2] (%mol)	Native[3]
Chondrus crispus	Female gametophyte	Portugal (Wild)	23.2 %	70.0κ, 28.0ι	κ - ι (μ/ν)
C. crispus	Tetrasporophyte	Portugal (Wild)	36.6 %	100.0 λ	λ
C. crispus	Tetrasporophyte	Canada (Cultivated)	43.6 %	100.0 λ	λ
Sigma	-	-	-	100.0 λ	λ
CP-Kelco	-	-	-	100.0 λ	λ

[1]expressed in percentage of dry weight; [2]composition determined by ^{1}H-NMR; [3]composition determined by FTIR-ATR and FT-Raman analysis of ground seaweed samples; the carrageenans are identified according to the Greek lettering system; the letters between parenthesis ( ) correspond to the biological precursors of the carrageenans, present in native samples.

2.4 FTIR-ATR and FT-Raman Analysis

The FTIR spectra of sample materials (ground dried seaweed, native and alkali-modified carrageenan) were recorded on an IFS 55 spectrometer, using a Golden Gate single reflection diamond ATR system, with no need for sample preparation. All spectra are the average of two independent measurements with 128 scans each at a resolution of 2 cm^{-1}.

The corresponding FT-Raman spectra were recorded on a RFS-100 Bruker FT-spectrometer using a Nd:YAG laser with an excitation wavelength of 1064 nm. Each spectrum was the average of two repeated measurements, with 150 scans at a resolution of 2 cm^{-1}.

2.5 NMR Analysis

^{1}H-NMR spectra were taken on a Bruker AMX600 spectrometer operating at 500.13 MHz at 65 °C. Typically 64 scans were taken with an interpulse delay of 5 s (T_1 values for the resonance of the anomeric protons of κ- and ι-carrageenan are shorter than 1.5 s). Sample preparation for the ^{1}H-NMR experiments involved dissolving the carrageenan sample (5 mg mL^{-1}) at 80 °C in D_2O containing 1 mM TSP (3-(trimethylsilyl) propionic-*2,2,3,3-d*$_4$ acid sodium salt) and 20 mM Na2HPO4, followed by sonication for three times 1 h in a sonicator bath (Branson 2510), according Pereira *et al.* (2007). Chemical shifts (δ) are referred to internal TSP standard (δ = -0.017 ppm) relative to the IUPAC recommended standard DSS for ^{1}H according to van de Velde *et al.* (2004). Assignments of the ^{1}H-NMR spectra were based on the chemical shift data summarized by van de Velde *et al.* (2002, 2004).

3 RESULTS AND DISCUSSION

The main results of the analyses are listed in Table 1. The assignments of the IR spectra were mostly based on the previous work of Chopin *et al.* (1999) and Sartori *et al.* (1997). The Raman spectra were assigned based on the IR information and on the comparison between samples of known composition, controlled by NMR spectroscopy.

The carrageenans are identified by the Greek lettering and by the letter code proposed by Knutsen *et al.* (1994).

Figure 1 presents four different FT-Raman spectra (*Chondrus crispus,* female gametophytes), corresponding to the different tests of depigmentation to reduce the background signal from unwanted laser-induced fluorescence in Raman

spectroscopy. The spectrum A corresponds to the ground seaweed treated with a mixture of acetone and methanol; this presents some fluorescence, particularly in the spectral area 600-875 cm^{-1} and the peaks are ill-defined. The spectrum B corresponds to the fresh seaweed treated with calcium hypochlorite 4% (30 s), then dried and milled. The spectrum C concerns to the ground seaweed (obtained from a herbarium sample) treated with calcium hypochlorite 4% (30 s). Finally, the spectrum D was obtained from the native carrageenan (*C. crispus* water-extracted) analysis. The last three spectra (B, C, D) don't present fluorescence, with peaks well-defined and without background noise.

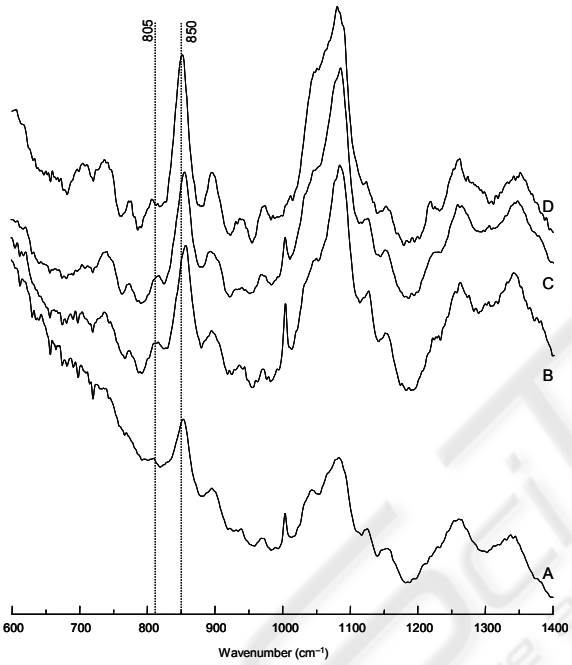

Figure 1: FT-Raman spectrum of ground seaweed (*C. crispus* female gametophyte) treated with a mixture of acetone and methanol (A). FT-Raman spectrum of fresh seaweed treated with calcium hypochlorite 4% (30 s), then dried and grounded (B). FT-Raman spectrum of ground seaweed (obtained from a herbarium sample) treated with calcium hypochlorite 4% (30 s) (C). FT-Raman spectrum of *C. crispus* extracted carrageenan (D).

Since this algae produces a hybrid kappa/iota-carrageenan the diagnoses peaks referenced in Figure 1 are the 805 cm^{-1} (DA2S),corresponding to iota-carrageenan and 850 cm^{-1} (G4S), corresponding to kappa-carrageenan.

The FTIR-ATR and FT-Raman spectra of commercial lambda-carrageenan (Sigma) and ground *C. crispus* tetrasporophytes are shown in Figure 2. These samples present high sulphate content as indicated by the broad band between 820 and 830 cm^{-1} in FTIR-ATR spectra. The *C. crispus* and lambda-carrageenan FT-Raman spectra show two combined peaks between 815 and 830 cm^{-1}.

Figure 3 shows the FT-Raman spectra of commercial sample (CP Kelco) of pure lambda-carrageenan (A), alkali-extracted carrageenan (B) of *C crispus* (tetrasporophyte) and ground seaweed sample (C) of *C. crispus* (cultivated strain). The spectrum of alkali-extracted carrageenan is similar to that of commercial pure lambda-carrageenan. The high sulphate content, typical of the lambda variant, is patent in the spectra, with a presence of two combined peaks at 815 cm^{-1} (G/D6S) and 830 cm^{-1} (G/D2S).

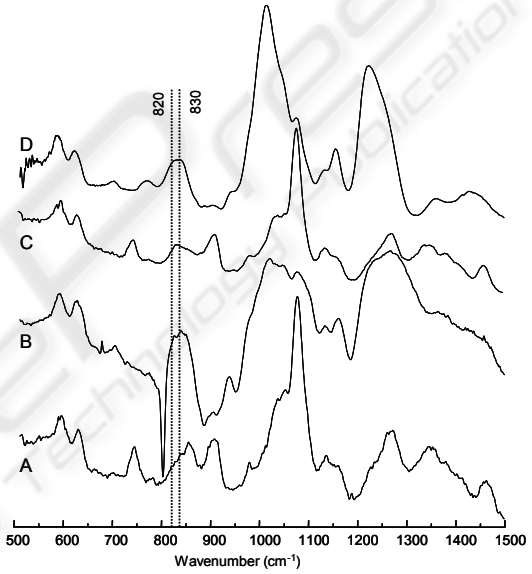

Figure 2: FT-Raman (A) and FTIR-ATR (B) spectra of commercial lambda-carrageenan (Sigma); FT-Raman (C) and FTIR-ATR (D) spectra of ground seaweed sample (*Chondrus crispus*, tetrasporophyte).

4 CONCLUSIONS

The present work confirms the usefulness of FTIR spectroscopy in the comparative study of carrageenan types. However, it also shows that the complementary use of IR and Raman spectroscopy provides relevant additional information, allowing a better interpretation of the vibrational spectra and a more accurate identification of diverse colloids and variants. In fact, due to the different selection rules, bands of weak intensity or even absent in the IR spectra may appear as sharp and intense bands in the Raman spectra. This is particularly evident, for instance, in the spectra of different fractions

belonging to the family of lambda-carrageenan (Pereira *et al.*, 2003) and the biological precursors of kappa and iota-carrageenan (mu and nu, respectively) (Pereira and Mesquita, 2004).

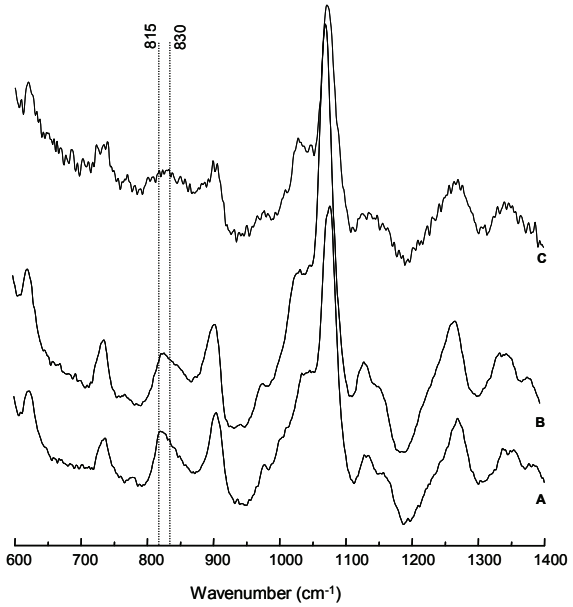

Figure 3: FT-Raman spectra: (A) commercial sample of pure lambda-carrageenan (CP Kelco); *Chondrus crispus* (tetrasporophyte) alkali-extracted carrageenan; (C) ground seaweed sample (*C. crispus*, cultivated strain).

With the combination of these two spectroscopic techniques (ATR-FTIR and FT-Raman), it is now possible the rapid and reliable identification of all major types of carrageenan, both extracted carrageenan and grounded material samples. The joint application of these spectroscopic techniques has as main advantages:

a) It is a quick and simple methodology in phycocolloid analysis. Only need few minutes, instead of several days needed for the extraction of colloids;

b) Requires small quantities of algal material (a few grams of weight fresh or milligrams of dry weight), allowing the analysis of herbarium samples, even of algae or portions of algae with small size;

c) Since the seaweeds are subject to a process of minimal manipulation and treatment (they are simply dried and ground), the determined composition represents, as accurately as possible, the natural composition of phycocolloid produced.

Since the vibrational spectrometers are now standard equipment in many Laboratories, the techniques described in this work are useful for the implementation of strategies of sustainable seaweed harvest, the evaluation of the natural seaweed composition with industrial potential, the evaluation and control of the quality of the different batches of algal material harvested and/or cultivated. These spectroscopic techniques are also useful to analyze the composition of pharmaceutical and cosmetic excepients.

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