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# CHARACTERISING BIOMATERIALS BY SPECTROSCOPY

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Spectroscopy offers a very powerful set of techniques for investigating biomaterials. The combination of modern sampling methods and some ingenuity make it possible to obtain useful information about structure and function in even very complex biomaterials. Examples are given of the applications of NMR, ESR and infrared spectroscopy to investigate such diverse materials as wheat gluten, chocolate, green tea and meat.

## INTRODUCTION

Biomaterials are hard to handle, typically they change irreversibly on heating or dehydration, are heterogeneous, chemically complex and often optically opaque. Spectroscopy is in principle ideally suited to the investigation of these materials in that it only interacts with the sample through radiation and can return information on the chemical and physical state of the system of interest. Whether one is interested in the behaviour of an individual component or of the interactions between components it is usually necessary to be able to discriminate between the components in the system. Additional complexity is added to the problem by the fact that a component may occur in a variety of physical states or in different environments within the same system and it may be a requirement to be able to discriminate between these as well.

A number of strategies are possible to deal with the problems and in practice a combination of methods are usually used.

The strategies may be listed as follows: (1) separate chemically; (2) use isotopes; (3) use spin probes; (4) separate spectroscopically by: chemical state, physical state, dynamic state.

In the following some examples of the applications of these strategies using NMR ESR and Infrared spectroscopy will be given.

## NMR METHODS

A simple illustration of the way in which a straightforward chemical separation combined with use of readily available stable isotopes may be used to study hydration is given by the problem of measuring the uptake of water by gluten. The hydration of gluten is intrinsically interesting because of its relevance to the water absorption of flour and because it has often been assumed in the literature that gluten is hydrophobic. By separating gluten from starch by washing (the chemical step) and submerging it in deuterium oxide it is possible to use deuterium NMR to observe the behaviour of the water only. The non-exchangeable protons on the protein, which represent the bulk of the material, will not contribute to the signal. Thus an editing process is achieved. However it is still necessary to discriminate between the bulk water and that associated with the protein phases. This may be done by careful measurement of the transverse relaxations time of the water [Grant *et al.*, 1999; Belton *et al.*, 1994] and interpretation of the data based on a careful theoretical analysis [Belton & Hills, 1987]. The results show that as the temperature is raised the amount of water associated with the gluten increases, exactly the reverse of that expected for a hydrophobic material, showing that gluten is indeed hydrophilic.

By using the same system and this time observing the proton resonance the signal arises from the unexchangeable protons of the protein. Figure 1 shows a typical signal observed as a decay of transverse relaxation. In this case the samples are of gluten which has been treated with different salts [Wellner *et al.*, 2003]. The fast decaying part corresponds to signals from solid like parts of the protein and the slow decaying parts from more mobile regions. As water is added the protein is plasticised and the mobile to immobile ratio decreases as shown in Figure 2. The ratio reaches a constant value at water to protein ratios greater than 1 demonstrating the saturation of the insoluble protein with water. It is significant that typically water to protein ratios in bread doughs are around 0.8 thus indicating the importance of controlled plasticisation in bread dough.

Muscle tissue is a very complicated biomaterial both in its viable form and as meat. In the production of sausages sodium chloride and tripolyphosphate are added to improve tenderness and succulence. In order to investigate the role of sodium chloride $^{35}Cl$ NMR was used [Belton *et al.*, 1987]. When the muscle was aligned such that the long axes of its sarcomeres were perpendicular to the magnetic field fine structure on the chlorine signal was observed that indicated

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that the chloride ions were constrained to anisotropic motion along the long axes of the sarcomeres, probably due to restrictions to motion causes by the actomyosin filaments. On treatment with tripolyphosphate the fine structure disappeared indicating that disruption of internal structure had occurred resulting in the formation of a random protein network.

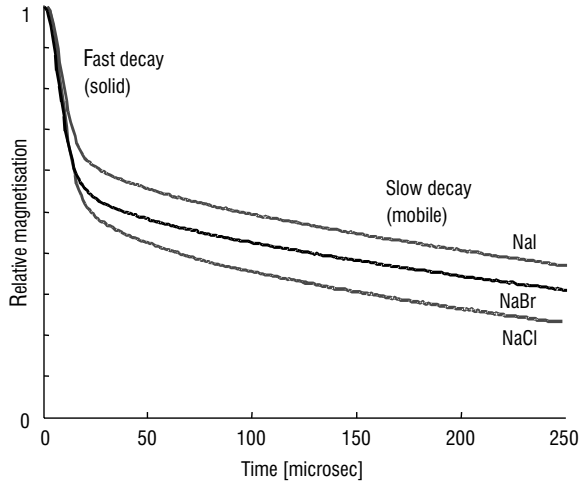


FIGURE 1. Free induction decays of gluten in the presence of different salts. Note the fast decaying immobile, solid, fraction and the slower decaying mobile fraction

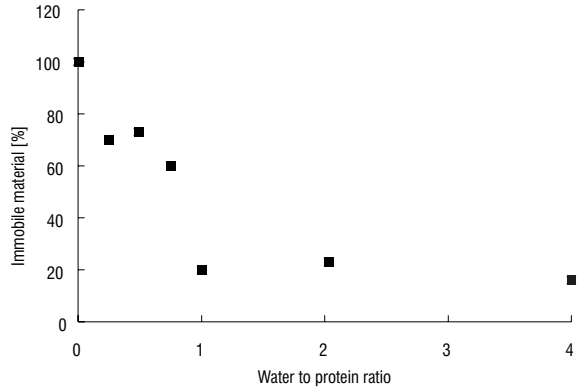


FIGURE 2. Variation in the amount of immobile material in the high molecular weight subunit proteins of wheat gluten as a function of water content.

One of the major developments in NMR over the past few years has been the great improvement in signal to noise ratios that can be obtained in solution state spectra. This can be exploited for the chemical characterisation of very complex mixtures in solution, typically proton NMR is used as this has the highest signal to noise ratio. Sensitivity on its own however is not enough since most systems of interest will have large quantities of water present an efficient method of suppressing the water signal is required. The spectra are of such quality that juices from different apple cultivars may be distinguished as shown in Figure 3. In some cases, for example alcoholic drinks, suppression of the alcohol signal is also needed. This can now be achieved routinely [Belton *et al*., 1997; Duarte *et al.,* 2002] and one dimensional spectra can be obtained in times of ten to fifteen minutes. When the one dimensional spectra are combined with some 2 dimensional spectra 28 separate compounds can be identified. However many more peaks are still unassigned. This does not limit comparison of spectra since even though assignment has not been completed the use of multivariate analysis permits the classification of the samples purely on spectral shape. A further refinement of the NMR method is to combine it with mass spectrometry in a combined NMR mass spectrometer allowing the direct mass spectrometric analysis of the samples subjected to NMR [Spraul *et al.*, 2001]. Under these circumstances further characterisation of the sample is possible. For example in beer a number of malto-oligosaccharides may be identified.

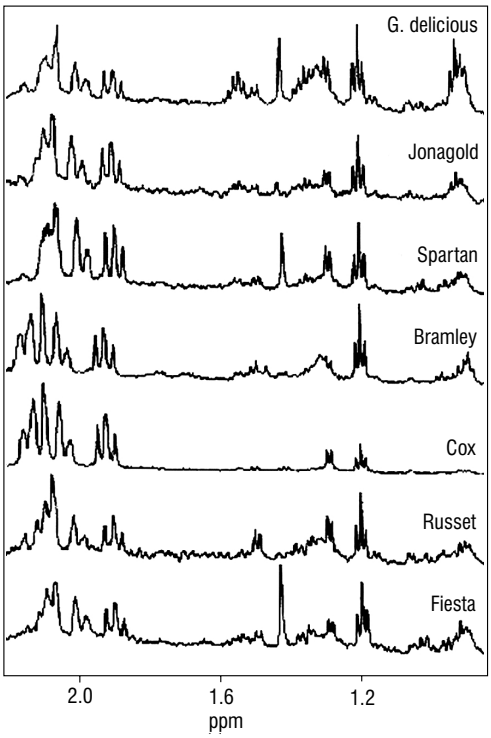


FIGURE 3. Part of the proton high-resolution NMR spectra of the juices from different cultivars of apples.

## ESR METHODS

Electron spin resonance can only observe signals arising from unpaired electrons, since these occur either in transition metals or organic free radicals there is an inherent selectivity in the technique. This can be exploited to use free radicals as probes for motion [Belton *et al.*, 1999] or chemical changes in complex systems. An illustrative case is the behaviour of antioxidants from green tea polyphenols in lipid vesicles. Such a system is a model for the behaviour of the polyphenols as they enter the cell. Since one of the sites for oxidative attack by free radicals is the cell membrane there is interest as to whether the polyphenols are able to act there or whether they only act on the aqueous phase. A spin label DPPH (1,1-diphenyl-2-picrylhydrazyl) is soluble in the lipid phase but not soluble in water. As a consequence the spin label only reports on behaviour in the lipid phase. When green tea polyphenols are introduced into an aqueous suspension of lipid vesicles (made from DHAPC, 1-hexadecanoy-2-[(cis,cis,cis,cis,cis

cis)-4,7,10,13,16,19-docosahexaenoyl]-sn-glycero-3--phosphocholine). The intensity of the spin label signals decreases indicating that the polyphenols have entered the lipid phase and are able to scavenge free radicals there. This demonstrates that not only are the antioxidant polyphenols active in the aqueous phase but also in the lipid phase and may thus carry out their protective role in the cell membrane [Chen *et al.*, 2000].

## INFRARED METHODS

In the last twenty years infrared spectroscopy has undergone a revolution. The widespread use of Fourier transform methods combined with a variety of new sampling methods had meant that hitherto intractable samples may now be routinely examined. One of the most important sampling methods is Attenuated Total Reflectance (ATR). In this a beam of light is passed through a high refractive index crystal such that it is totally internally reflected (Figure 4). The sample is situated at the surface where the reflection is taking place. Energy may pass from the light beam to the sample at the point of reflection if the sample absorbs at the wavelength of the light. The light entering the sample is called the evanescent ray. Typically the effective distance over which the interaction takes place (the penetration depth) is of the order of a micron. The apparatus is thus effectively a very short path length cell in which the actual thickness of the sample is not relevant, provided it is greater than the penetration depth. It is therefore possible to obtain high quality spectra from almost totally opaque materials such as chocolate [Belton *et al.*, 1988]. In this case it is possible by examining the detail of the carbonyl peak around 1750 $cm^{-1}$ to observe the formation of a number of crystalline phases as the melt solidifies.

The problem of starch retrogradation is one that continues to be of interest in a variety of contexts. The infrared spectrum of starch is very sensitive to the degree of crystallinity of the sample and can thus be used for both following gelation and retrogradation. By following the retrogradation process quantitatively it was possible to determine the degree of crystallinity of the system and from that predict the rheological properties of the sample [Wilson *et al.*, 1987].

The role of proteins in the stabilisation of interfaces is one that is of interest both from the biological point of view and because proteins may be able to replace synthetic emulsifiers in cosmetics and foods. Whilst the ATR method is very valuable, there are limitations when it comes to the study of interfaces since the only interface that is sensed by the light absorption is the sample crystal interface. In order to study, for example, air water interfaces direct sensing of the interface is required. This is possible but difficult. To be useful the surface pressure of the air water interface must be measured at the same time as the infrared spectrum since it is likely that protein conformation is sensitive to surface pressure. This may be done by building a Langmuir trough, onto the surface of which the incident beam is directed the apparatus must also be fitted into the sample chamber of an infrared spectrometer and the absorption due to water vapour above the trough controlled. The incident light is reflected of the surface after passing through the protein layer, it thus contains information about the absorption of light by the proteins. Because of various optical effects due to reflection the spectrum is inverted but is readily comparable with spectra from protein solutions. The most diagnostic band for structure is the amide I band centred around 1650 $cm^{-1}$. The results for beta lactoglobulin show [Burnett & Belton, 2002] that in solution the spectrum indicates that the structure is largely alpha helical but that the protein loses alpha helix and becomes much richer in beta sheet structure. This is probably due to partial denaturation of absorption followed by coagulation at the surface.

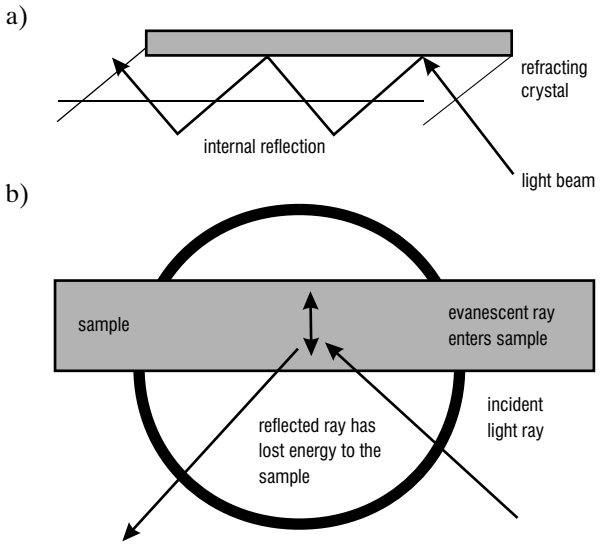


FIGURE 4. The principles of attenuated total reflectance infrared spectroscopy. For explanation see text.

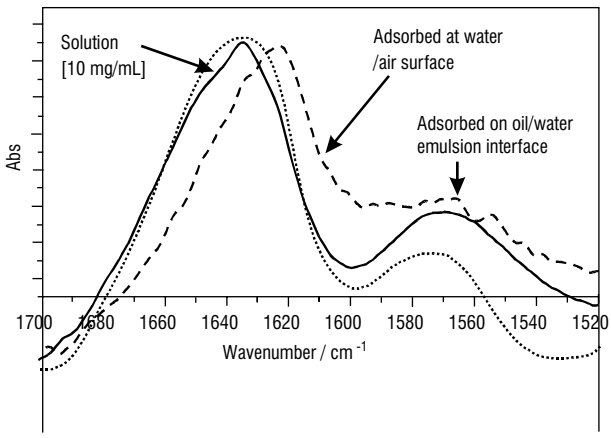


FIGURE 5. Infrared spectra in the Amide I and II regions of beta lactoglobulin in solution, at the air water interface and the oil water interface.

## CONCLUSION

Spectroscopy offers a wide variety of methods for the exploration of biomaterials. The examples given here are by no means comprehensive but it is hoped that they do indicate that an appropriate choice of spectroscopy coupled with a willingness to approach apparently intractable materials can bring useful results.

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