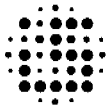

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Using Protein Hydrolysates as Raw Materials for Serum-Free Cell Culture Media

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ABSTRACT

Protein hydrolysates have long been used in serum-free cell culture media as supplements to improve growth or production. In many cases, protein hydrolysates have been used successfully as substitutes for less desirable media components, such as animal serum, or serum-derived proteins. As requirements for serum-free culture media have become more stringent, hydrolysates have played an important role in facilitating the development of low-protein media, protein-free media, and media that are free of animal-derived-components. While the use of hydrolysates has allowed these stepwise improvements in media over the years, it is important to also recognize the many issues that their use may present to the development of a sound manufacturing process for therapeutics and how those issues can be effectively addressed.

Issues

- **Raw Material Sources**

The source of raw materials used in the manufacture of protein hydrolysates is of fundamental importance to be able to identify and control several important quality parameters. While it may be important to know the complete phylogeny of the starting materials, certainly, it is most critical to know whether the materials are animal-derived or not. Animal-derived protein hydrolysates have been widely used in the past, but because of the associated risks, are generally considered unacceptable for the development of new therapeutic production processes today.

Bulk Material - Animal or Plant

While hydrolysates made from bovine and other animal species have been commonly used in the past, more recent efforts have focused exclusively on protein hydrolysates derived from non-animal sources. Among the sources of bulk protein commonly available are:

Rice	Soy
Wheat	Pea
Potato	Corn
Cotton Seed	

Protease - Animal or Plant

Even when the bulk materials are from a certified non-animal source, it is not uncommon for hydrolysates to be produced using porcine-derived trypsin, or other animal-derived proteases during the digestion process. It is therefore important to know about the entire hydrolysate production process, and specifically about the sources of any enzymes used during the digestion process.

- **Protease inactivation**

Another important aspect of the protein hydrolysate production process is the inactivation of any proteases used during digestion. Because active proteases could cause serious problems if introduced into the manufacturing process of a therapeutic, it important to ensure that all proteases are completely inactivated before any hydrolysate is used as a component of a cell culture medium. Inactivation can usually be accomplished either chemically, or by exposing the digest to high temperatures.

- **Consistency**

Poor consistency is often mentioned as one of the major objections to the use of hydrolysates in a cell culture process. The consistency of hydrolysates can, however, be controlled to a large extent, through a combination of approaches:

1. Carefully evaluate several lots from several supply sources before choosing an approved source of raw materials
2. Incorporate incoming specifications that will effectively screen lots for desired characteristics, including cell culture performance. FTIR spectrometry can be useful to measure the amount of variation between lots. (see figures 1-4)
3. Perform further processing that will eliminate variable components of hydrolysates. Ultrafiltration, for example, provides an effective method for removing variable high molecular weight components (see Ultrafiltration below).

- **Contaminants**

Another objection to the use of hydrolysates in a cell culture process that is often mentioned is the risk associated with various possible contaminants:

Pathogens

It is because of the risk of contamination with human or animal pathogens that hydrolysates from non-animal sources are preferred. Using hydrolysates that are produced completely from non-animal materials and using processes that effectively eliminate any exposure to animal materials help to minimize the risk of pathogen contamination.

Pesticides, Alkaloids, etc.

It may be important in some applications to prove that other possible contaminants, such as pesticides or alkaloids, are not detectable in hydrolysates that are plant derived.

Endotoxin

The concentration of endotoxin in hydrolysates is often unacceptably high for use in manufacturing therapeutics. Ultrafiltration can effectively reduce endotoxin levels of hydrolysate solutions (see Ultrafiltration below).

Residual protein

Although hydrolysates consist primarily of small peptides, they may also contain significant amounts of residual protein. This residual protein may be either the result of incomplete digestion of the bulk protein, or small amounts of remaining inactivated protease.

- **Solubility**

As with all other culture media components, it is important that any hydrolysate used in a cell culture process be completely soluble. A wide range of hydrolysates is available, including many with relatively poor solubility. Solubility is important not only for achieving good homogeneity in the culture vessel, but also to avoid problems with membrane filtration of the medium.

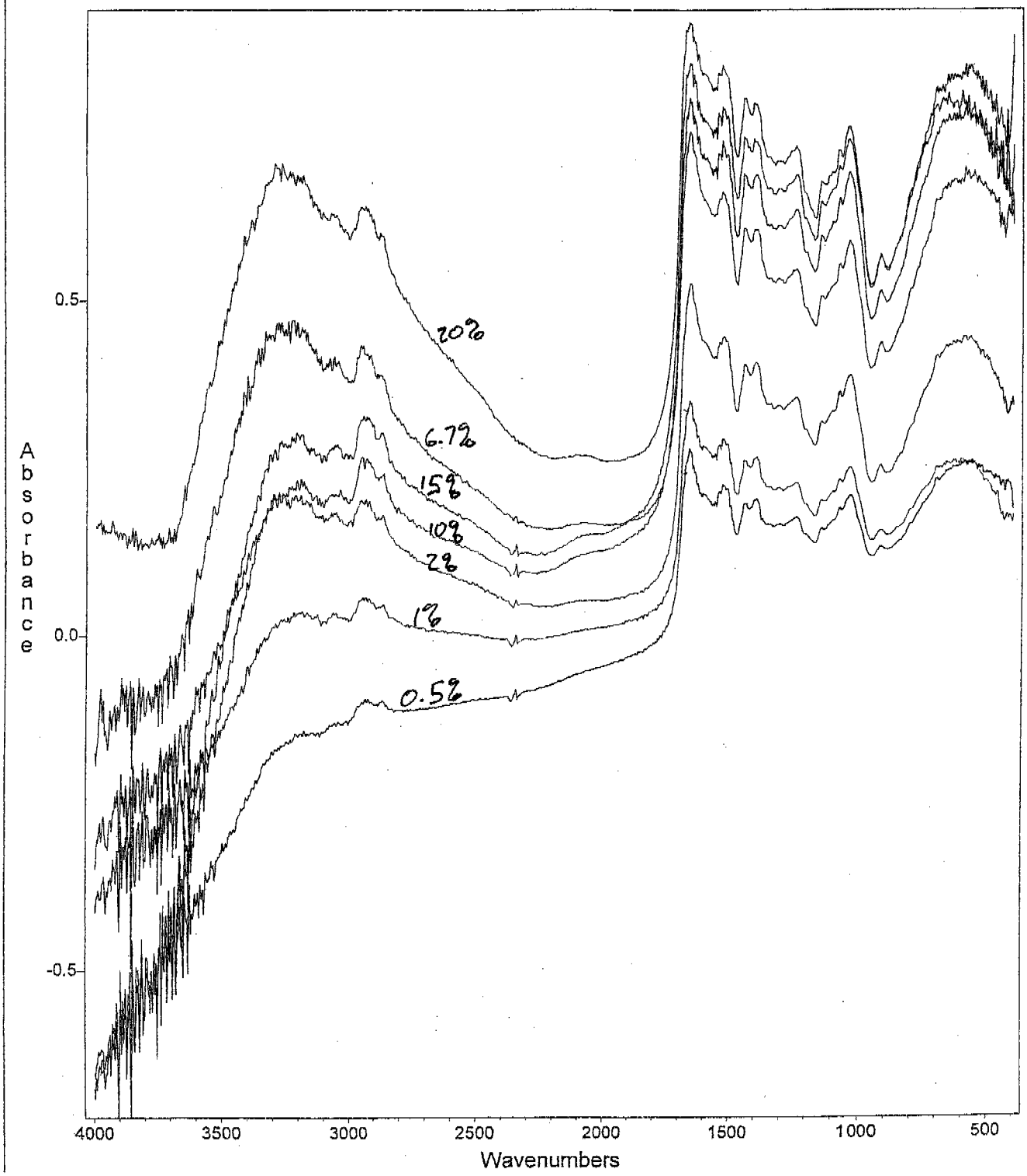


Figure 1. FTIR Method Development. Because of the complex composition of hydrolysates, special methods were developed for Diffuse-Reflectance FTIR matching. Besides customizing the matching algorithm, the sample:KBr ratio was optimized to best exploit the special features of hydrolysate spectra. The figure demonstrates the effects of adjusting the sample:KBr ratio over a range from 0.5-20%.

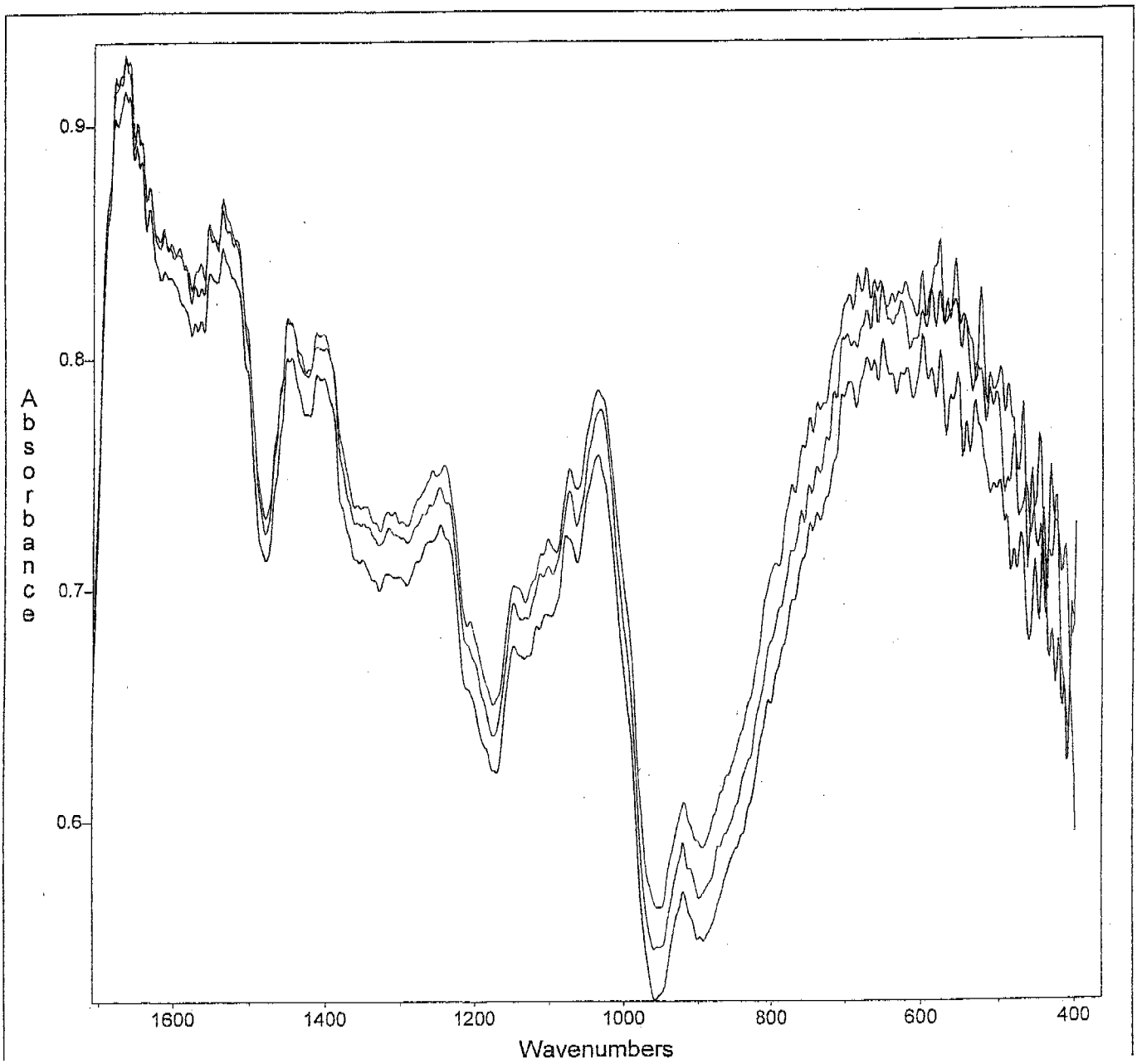


Figure 2. FTIR Method Reproducibility. Three independent samples of the same lot of hydrolysate were analyzed to demonstate the reproducibility of the FTIR analysis method. The samples were analyzed at a 20% sample:KBr ratio.

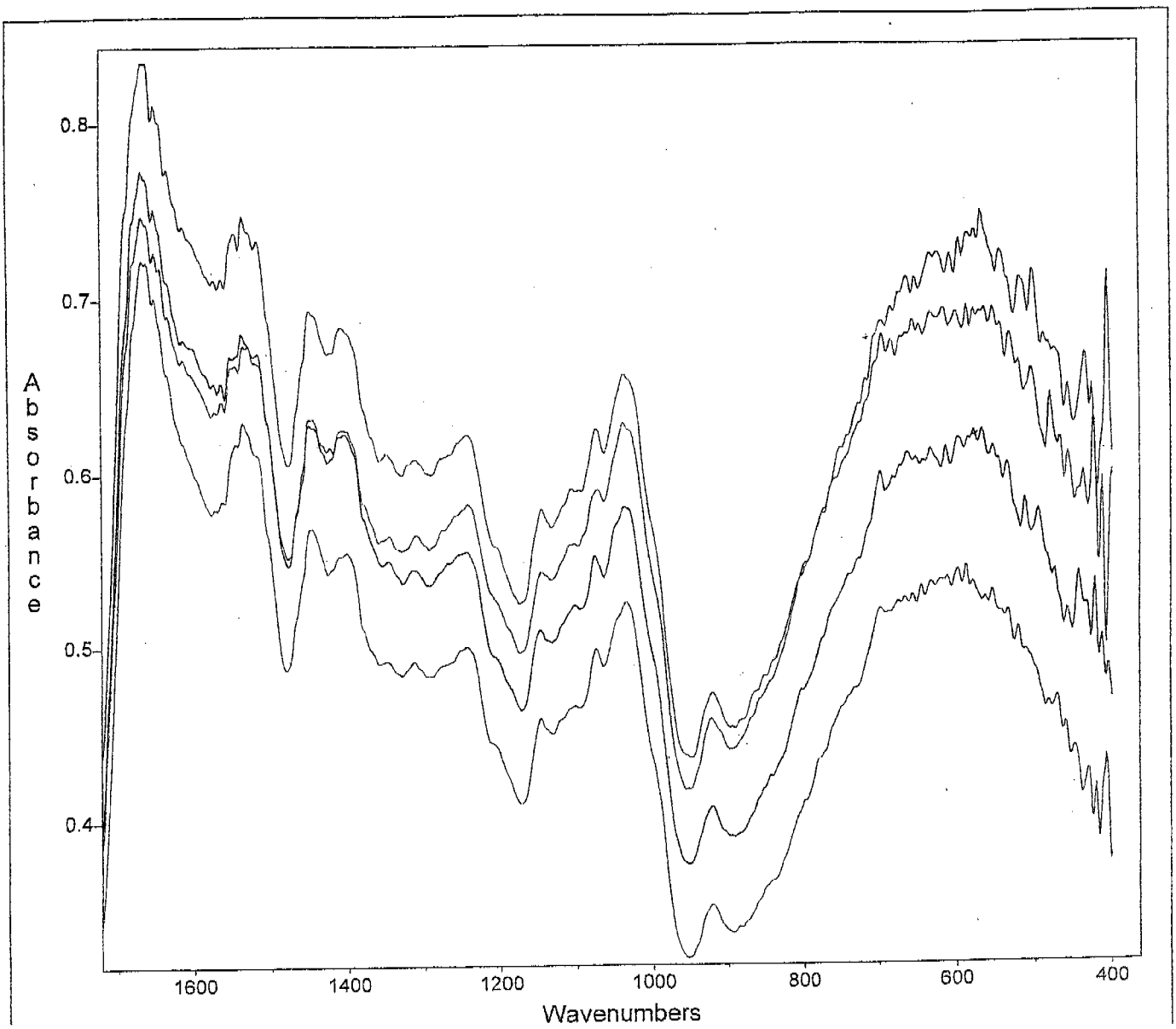


Figure 3. Lot-to-Lot Consistency. Four separate lots of hydrolysate C were analyzed. These results demonstrate excellent consistency between the lots.

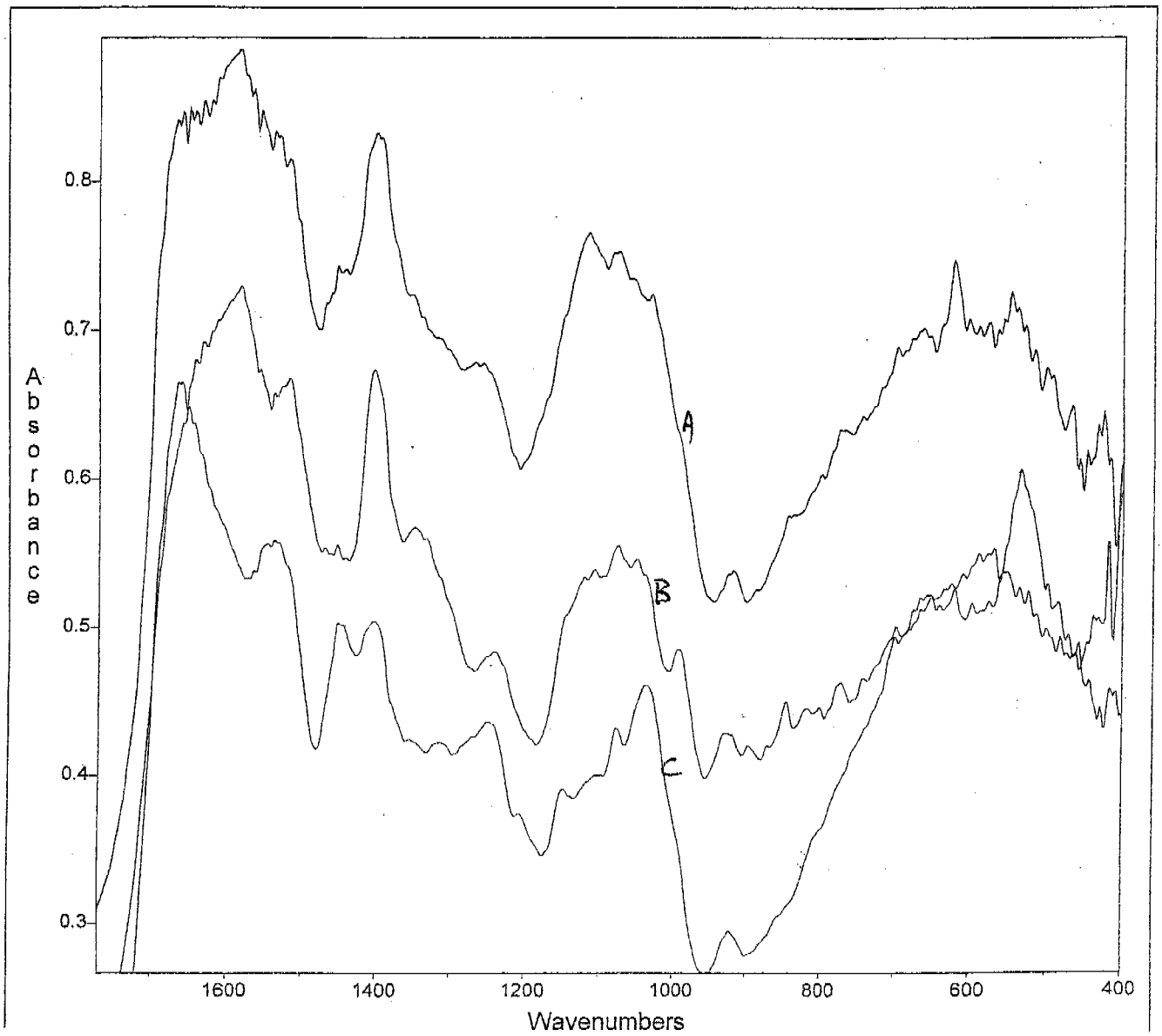


Figure 4. Distinguishing Various Hydrolysates. This figure demonstrates how an FTIR method can effectively distinguish between different hydrolysates. The spectra acquired from hydrolysates of three different non-animal sources are shown.

Solutions

Several of the important issues associated with the use of hydrolysates in a therapeutic manufacturing process have been resolved using the following practical approaches:

- **Ultrafiltration**

Ultrafiltration of a hydrolysate solution effectively removes components above the specific molecular weight cut-off (MWCO) of the ultrafiltration membrane used. Since several of the issued related to the use of hydrolysates are linked to the presence of these various high molecular weight components, ultrafiltration can help to resolve these issues.

1. Endotoxin is effectively reduced
2. Residual proteins (including proteases – active or inactive) are removed
3. Consistency is enhanced by removal of variable high molecular weight components
4. Any high molecular weight contaminants or pathogens are removed

- **Blending**

In many cases, a single protein hydrolysate cannot provide nearly the same benefits to cell culture performance that can be achieved by using a blend of different hydrolysates. Different hydrolysates may even have distinctly different benefits for the culture.

- **Testing and specifications**

By using carefully designed raw material testing and specifications, the consistency of hydrolysates can be ensured. Hydrolysates can be tested on a lot-by-lot basis for specific chemical parameters, such as fingerprinting by FTIR spectrometry, as well as for performance in cell culture assays.

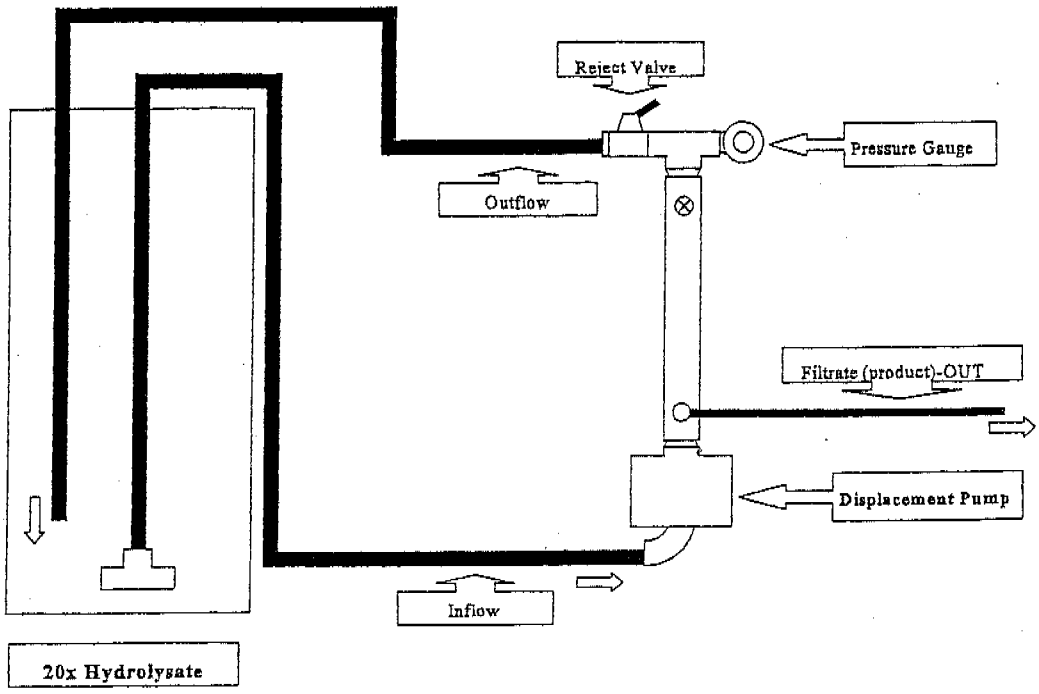


Figure 5. Ultrafiltration of Hydrolysates. A concentrated solution of dissolved hydrolysates may be ultrafiltered and subsequently dried for use in culture media. A 10,000 MWCO membrane is most commonly used

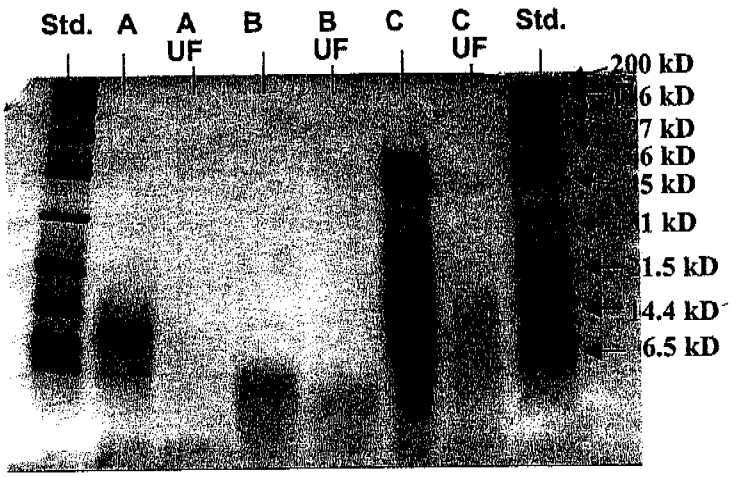


Figure 6. Effects of Ultrafiltration. This SDS-PAGE gel shows the effectiveness of ultrafiltration for removing residual protein from a hydrolysate solution. Three different hydrolysates (A, B, and C) were each analyzed with and without processing by ultrafiltration. Samples were dissolved in deionized water and filtered by a 0.2 μm membrane before ultrafiltration. 20 μL of sample was applied to each well of a 10-20% Tris-Tricine Gel (Bio-Rad) after mixing 1:2 in SDS sample solution.

How do Hydrolysates work?

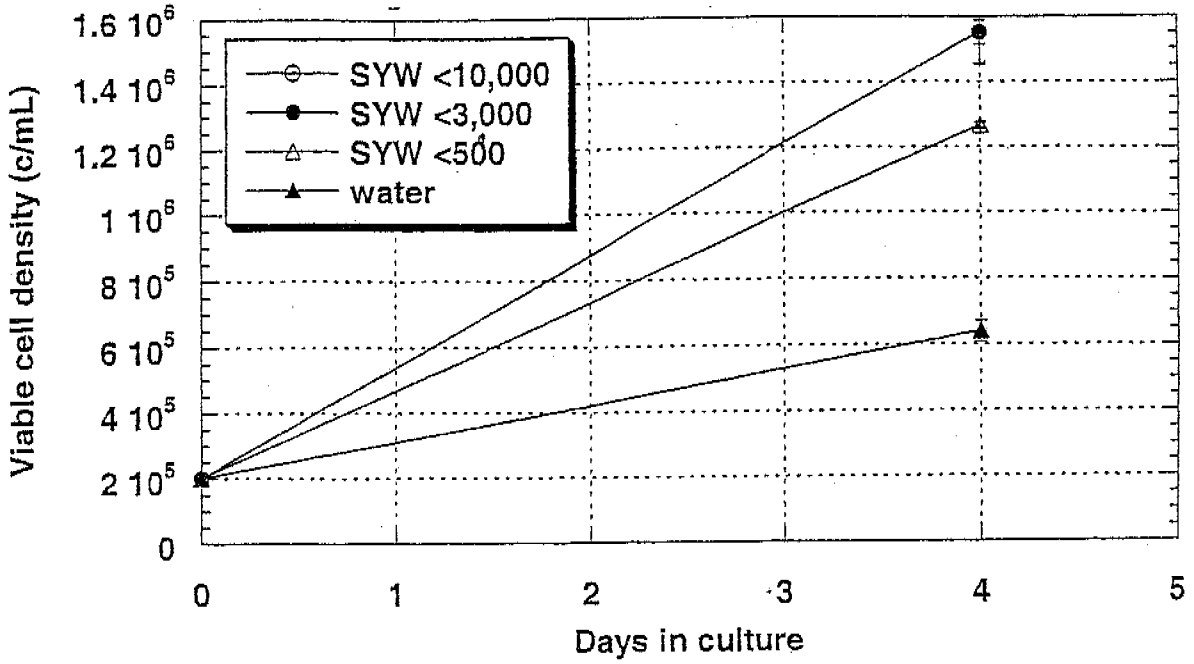
In the search for chemically defined alternatives for protein hydrolysates, several studies have attempted to learn more about the mechanism by which hydrolysates provide benefits to cell cultures.

- **Fractionation**

Fractionation studies have demonstrated that effective components of protein hydrolysates exist even below 500 daltons.

- **Synthetic Peptides**

Recently studies performed using synthetic peptides have demonstrated that cell culture performance can be enhanced by specific pure peptides. These studies have also demonstrated that, while the peptides were effective in promoting culture performance, the peptides themselves were not consumed by the cultures. These results imply that the peptides are providing a benefit other than simple nutrition for the cultures.



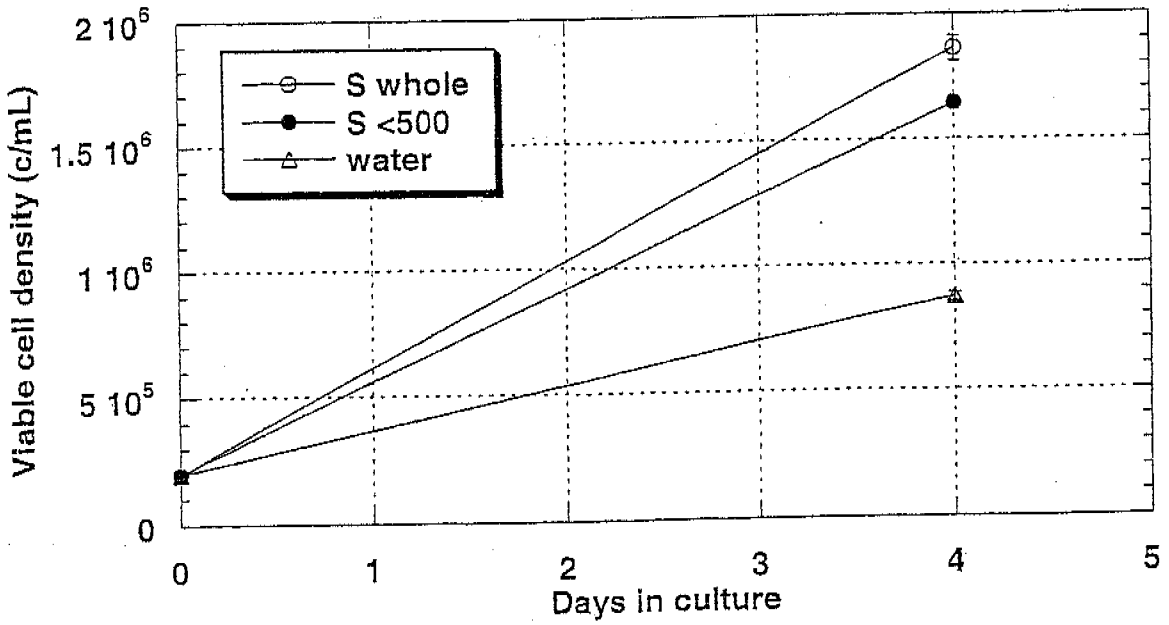


Figure 7. Performance of Hydrolysate Fractions in Cell Culture. Chinese Hamster Ovary (CHO) cell cultures were grown in Duplicate 125 mL shaker flasks in a medium supplemented with various MW fractions of hydrolsate solution. Water was substituted for the hydrolysate solution as a control. The results show that essentially all of the growth promoting activity of the hydrolysate is contained in the < 3,000 MW fraction, and most of the activity exists even in the < 500 MW fraction.

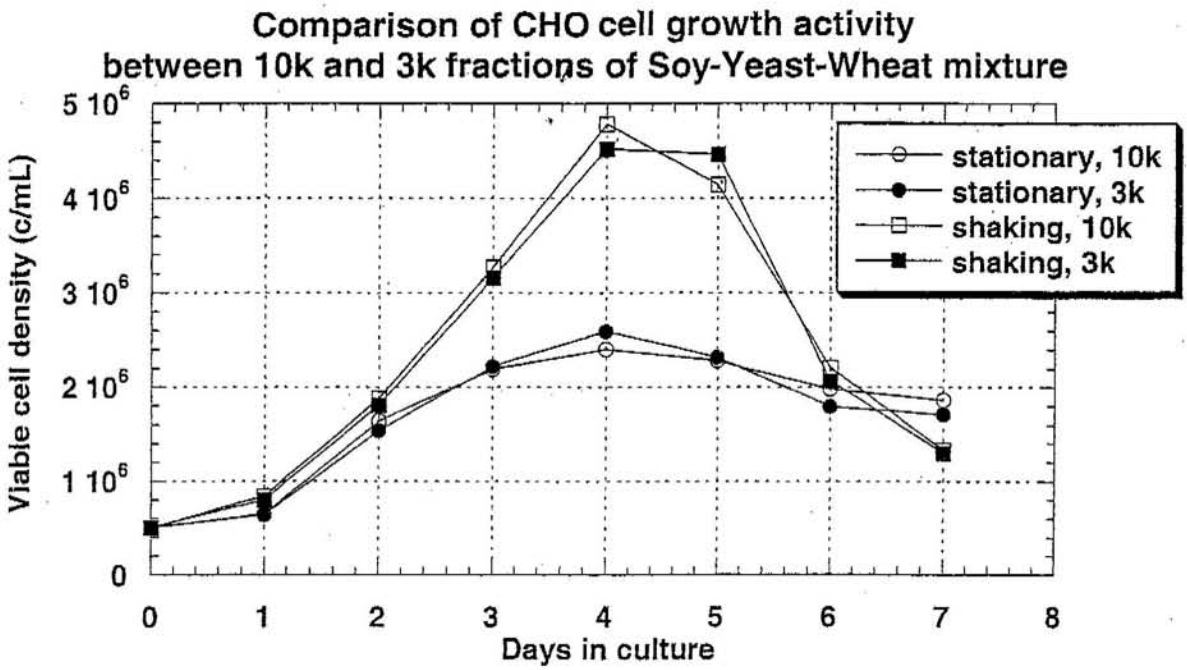


Figure 8. Comparison of Cell Growth Activity. Two hydrolysate MW fractions, < 3,000 MW and < 10,000 MW, were directly compared in both stationary cultures and shaker cultures. The results clearly confirm that the active components of this hydrolysate are less than 3,000 MW.

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