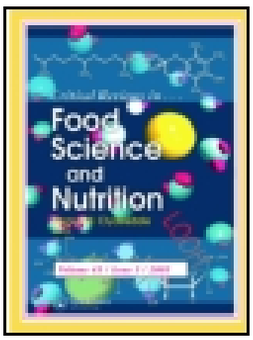

Critical Reviews in Food Science and Nutrition

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/bfsn20

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To cite this article: Ming Yan , Víctor H. Guevara-Oquendo , María E. Rodríguez-Espinosa , Jen-Chieh Yang , Herbert (Bart) Lardner , David A. Christensen , Xin Feng & Peiqiang Yu (2020): Utilization of synchrotron-based and globar-sourced mid-infrared spectroscopy for faba nutritional research about molecular structural and nutritional interaction, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2020.1843397

To link to this article: https://doi.org/10.1080/10408398.2020.1843397

Published online: 28 Nov 2020.

CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION
https://doi.org/10.1080/10408398.2020.1843397

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REVIEW

Utilization of synchrotron-based and globar-sourced mid-infrared spectroscopy for faba nutritional research about molecular structural and nutritional interaction

Ming Yan, Víctor H. Guevara-Oquendo, María E. Rodríguez-Espinosa, Jen-Chieh Yang, Herbert (Bart) Lardner, David A. Christensen, Xin Feng†, and Peiqiang Yu

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ABSTRACT

The traditional wet chemistry analysis is to use combination of specific chemical reactions to quantify a group of compounds with similar chemical and nutritional properties. However, plant cell wall complex is not uniform in terms of chemical, physical or nutritional characteristics and the digestion progress is achieved by a series of enzymatic hydrolysis of specific chemical bonds which cannot be revealed by wet chemistry analysis. Synchrotron-based and globar-sourced mid-infrared spectroscopy instead utilizing the unique absorption of mid-infrared light at different frequencies and more information about specific chemical bonds can be revealed. As a result, taking spectral change during digestion into consideration may give some insight about nutritional utilization features. However, the utilization of synchrotron-based and globar-sourced mid-infrared spectroscopy on feed and food nutritional research is limited. Therefore, the aim of this study is to provide idea about how to systematically study the nutritional and spectral structure feature of faba bean with traditional and advanced synchrotron-based and globar-sourced vibrational molecular spectroscopy. The study reviews (1) Utilization of faba bean for human and animal consumption; (2) Traditional evaluation methods for faba bean nutritional characteristics and (3) Contribution of synchrotron-based and globar-sourced mid-infrared (Mid-IR) spectroscopy techniques to evaluate faba bean structural and molecular properties.

KEYWORDS

Vibrational molecular spectroscopy; chemistry and structure; nutritional availability; faba utilization; nutrition and structure interaction

Utilization of faba bean for human and animal consumption

History of faba bean production

Faba beans (*Vicia faba* L.) are also known as horse bean, broad bean, windsor bean. It belongs to the oldest crops in the world and it has been planted around the world for thousands of years (Singh et al. 2013). Major differences can be found between V. *faba* and other species belonging to the *Narbonensis* complex (*V. narbonensis, V. galilea, V. johannis,* and *V. hyaeniscyamus*) (Zohary and Hopf 1973). Although where the faba beans originally from is still under debate, it is generally agreed that *Vicia faba* L. was geographically originated from Near East and the subspecies V. *faba paucijuga* is its original form (Duc 1997). 5000 years ago, faba bean was used as a food resource in China and 2000 years later, it was cultivated by Egyptians, after that it was imported to Greeks and Romans (Singh et al. 2013). Nowadays, faba bean has been dispersed globally and mainly used as a food resource in industrialized countries including China, Ethiopia and Egypt, and China is the country with the highest production at 2×10^6 ha per year (Duc 1997). But in the United States, Canada and northern Europe, faba bean is planted mainly as a feed resource (Duc 1997; Singh et al. 2013). In 2006, the global planting area of faba bean reached 2.6 million ha and most of the producing area was in China, followed by Ethiopia and Europe (Stat 2009; Jensen, Peoples, and Hauggaard-Nielsen 2010).

In Canada, the first commercial production of faba bean was in 1972; after that year although the yield fluctuated, the general trend was upward. Before the 21st century, the content of anti-nutritional factors in faba bean is the main reason of relatively low production of faba bean. In 2002, the progress made in plant breeding leaded to the tannin-free genotypes, making faba bean more acceptable for both food and feed industries. After that, faba bean was widely planted in western Canada especially in provinces of Alberta, Saskatchewan and Manitoba (Duc et al. 1999; Oomah et al. 2011). In 2012 the cultivation area in western Canada provinces was less than 20 thousand acres, however, in 2015 the cultivation area in Saskatchewan and Alberta soared to more than 70 thousand acres (Phelps 2017). In Saskatchewan two types of faba beans are mainly cultivated and they are distinguished by the content of tannin. The

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tannin varieties of Taboar, CDC Fatime, Malik (FB9-4), CDC SSNS-1, Florent, Fabelle (low vicine) and Vertigo with brown seed coat and black dot have 8% to 9% of tannin. The low tannin varieties are Snowdrop, Snowbird, Imposa, and Tabasco with 1% of tannin and they have white flower and cream seed coat.

General information about faba bean

Plant and seed characteristics

The *Vicia faba* grows straightly with thick and erected stem to hold the height of the plant up to 1 to 1.5 m and the robust tap root branches with plenteous secondary roots spreading 0.6 m underground; the leaves of faba bean are made up of two to six not twining or slightly curled leaflets; faba bean flowers are prolific with white purple or pink colors and only one to six pods are produced on clusters (Hanelt and Mettin 1989). Pods of faba bean contain two to eight seeds and are up to 10 centimeters long, 1 to 2 centimeters wide. When mature, the color of pods and seeds get darker. Seeds weight per thousand seeds usually range from 400 to 800 grams, and food market usually demands seeds with size more than 650 grams per thousand seeds (Saskatchewan Pulse Growers 2020).

Growing characteristics

Faba bean is an annual legume and the optimal planting environment is well-drained loam or clay soils (Jensen, Peoples, and Hauggaard-Nielsen 2010). Faba bean is not suitable for being planted in light sandy soil. Normally, it flowers at 45–60 days after planting and the harvest period reaches around 70 days after flowering (Phelps 2017). The *Vicia faba* can tolerate cold weather up to −15 °C, but during its flowering, it is sensitive to heat and dry and the ideal annual precipitation for faba bean cultivation is around 650–1000 mm (Phelps 2017). Faba bean can tolerate most of the soil types and medium textured soils with pH between 6.5 and 8.0 is most suitable for its production (Phelps 2017). Moreover, faba bean is more resistant to acid soil type than most legumes (Hekneby, Antolín, and Sánchez-Díaz 2006; Idris 2008; Singh et al. 2013). In addition, faba bean has high requirement of P because of strong ATP demand. Before seedlings is fully accomplished, faba bean is sensitive to water supply, and it takes more than 20 days to emerge (Oplinger et al. 1989). The main pollinators of faba bean are bumblebees and around 35% of faba bean are cross-fertilized (Stoddard and Bond 1987).

Besides being use as food and feed resource, faba bean also shows its advantage in fixing atmospheric nitrogen by symbiosis with *Rhizobium* bacteria under broad climate conditions and crop rotations (Köpke and Nemecek 2010). Faba bean benefits subsequent crops by maintaining N pool in the soil at high level. Although the high N-fixing ability, faba bean should not be planted in the same area more frequent than every fourth year or be planted after consequent year of other pulses to avoid root and stem rot diseases. In addition, faba bean is also vulnerable to pests (Jensen, Peoples, and Hauggaard-Nielsen 2010).

Nutritional features

Faba bean has seeds rich in protein ranging from 247 to 372 g/kg DM in genetic resources and the protein contents trait is highly heritable (Phelps 2017). Starch is another main nutrient in faba bean seeds; however, it has negative correlation with protein content, with mean content of 423 g/kg DM (Duc et al. 1999). In addition to be used as a protein source, the N-fixing ability of faba bean demonstrates its potential use in sustainable cropping systems (Jensen, Peoples, and Hauggaard-Nielsen 2010).

With different single seed weight, two types of varieties are distinguished. V. faba major or broad beans are cultivars with large flatten seeds weighting from 1 to 2 g DM per seed while V. faba minor or field beans or horse beans are cultivars with smaller and round seeds weighing from 0.4 to 0.8 g DM per seed (Crépon et al. 2010). However, faba bean contains anti-nutritional factors concerning both human and animal nutrition. As for animal nutrition, the seed of faba bean contains vicine and convicince that have been demonstrated to have anti-nutritional effects on the growth of monogastric animals in several studies (Grosjean et al. 2001; Crépon et al. 2010). As for human health, faba bean seeds have been found to contain divicine and isouramil that are deleterious to people who carry a common genetic defect, to specify, faba bean can cause Favism which is an acute hemolysis in G6PD-deficient human individuals (Crépon et al. 2010).

Faba bean as human food

Faba bean has been extensively used as food resource in middle-east, Africa, and part of Asia, especially in China, which contributes to the majority of the world faba bean consumption. Seeds of faba bean, because of its high-quality protein and starch sources and various vitamins and minerals compositions, are mainly used as green vegetable in these countries. In sub-tropical and temperate regions, faba bean ranks the fourth important legume after dry beans, dry peas and chickpeas (Alghamdi 2009). For food market, faba bean cultivars (broad bean) with larger seed size are mainly consumed. The global broad bean production is around 5 million tons and China production accounts almost half of it. The major reason for the hugest consumption of faba bean in China is partly because of the popular of Sichuan cuisine around the country; fermented faba bean is the most important constituent of a spicy source-Douban Jiang that commonly used in Sichuan cuisine. As a result, faba bean is of huge demand in China. The second largest country of faba bean production is Ethiopia, and around 600,000 tons of faba bean is produced every year (Government of Saskatchewan 2020) Globally, the largest faba bean importer is Egypt, with around 500,000 of import every year; and the largest exporters are Australia, France and UK.

Main anti-nutritional compounds in faba bean

Although faba bean is a great source of high quality carbohydrate, protein, minerals and vitamins, its nutritional values can be offset by the anti-nutritional factors such as phytic acid, α-galactosides, trypsin inhibitor, lectin, pyrimidine glucosides (vicine and convicine) and condense tannins (under certain circumstances).

Tannins are secondary metabolites widely distributed in various plants protecting plant from pesticide predation and involving in plant growth regulation. Tannin is located in seed hulls of faba bean. Normally, tannin needs to be hydroxylated and polymerized (with molecular weight more than 500) to be capable of sufficiently binding with protein and other polymers to become a stable complex under desired chemical environment (Sinha and Amresh 2018). According to its hydrolytic ability, two classes of tannins, hydrolyzable tannins and condense tannins are identified (Rodríguez-Espinosa et al. 2020). Hydrolyzable tannin are readily degradable, and its degradation product is gallic acid which does not impact animal performance. Condense tannins are polymers of flavonoids, and the adverse effects of condense tannins to animals are because its astringency affects feed intake and its binding ability with protein affects protein digestion and absorption thus decreasing feed values (Sinha and Amresh 2018). However, according to the research of He et al. (2019), for ruminant diets with high protein degradation rate, less than 5% of total DM intake of condense tannins is able to avoid extra degradation of protein in the rumen and waste of high quality feed protein as ammonia. As a result, moderate tannin content in diets is advantageous rather than harmful for ruminants.

Phytic acid is a six-fold dihydrogen-phosphate ester of inositol. Phytic acid interacts with protein to prevent protein from effective digestion and it is also found to lower mineral availability in non-ruminants. However, in ruminants, microorganisms in the rumen are able to hydrolyze it, microorganism in the rumen is able to hydrolyze it (Sharma and Sehgal 1992).

α-galactosides are soluble, non-reducing sugars widely distributed in plant storage organs. They are the second most abundant soluble sugars in plants, being considered as sucrose derivatives (Martínez-Villaluenga, Frias, and Vidal-Valverde 2008). The main adverse effect of α-galactosides in faba bean is because of its high proportion of hindgut fermentation which provokes flatulence in monogastics (Martínez-Villaluenga, Frias, and Vidal-Valverde 2008).

Vicine and convicine (VC) are pyrimidine glycosides presenting in the cotyledons of faba bean; their metabolites in the small intestine divicine and isouramil can arise favism for people who has genetic deficiency in their red bloods cells (Gutierrez et al. 2006). In animal nutrition, VC are found to decrease apparent metabolizable value of faba bean in broiler chicken and affect egg quality and egg production rate in laying hens (Gutierrez et al. 2006). For swine, its negative effect seems to be negligible.

Trypsin inhibitors from faba bean seeds induce release of cholecystokinin in the gut leading to a major loss of S-containing amino acids, which in turn causes pancreatic hypertrophy and depression of growth (Savelkoul, Van Der Poel, and Tamminga 1992). Lectins are glycoproteins binding with specific proteins and sugars to cause agglutination of red blood cells (Savelkoul, Van Der Poel, and Tamminga 1992).

Faba bean use as feed

Use in monogastrics

Alternate legume sources have been extensively tested for possibility of substituting soybean meal (the most commonly used protein sources for pigs) in order to acquire more flexible and profitable feeding management. Faba bean seeds which are good source of protein, are also under consideration. The anti-nutritional content of condense tannin is the major concern for faba bean inclusion in the diet, and according to the research, the condense tannin of faba bean in pigs diet have adverse effect on protein utilization by increasing endogenous protein excretion and decreasing dietary protein digestion (Jansman et al. 1995). For faba bean with low or zero tannin contents, it was found that inclusion of zero tannin faba bean up to 30% does not adversely affect average daily gain or average daily feed intake but a reduced feed efficiency in the grower phase and reduced lean thickness were observed (Zijlstra, Lopetinsky, and Beltranena 2008). Similarly, inclusion of faba bean and peas for up to 30% in finisher and grower diets does not affect carcass quality and overall performance, although slight reduce of growth rate in finisher pigs (Smith et al. 2013). Although the possibility of including faba bean in pig diets is proved, the digestibility of major limiting amino acids is still lower than that of soybean meal, but it can be improved by pretreatments of extrusion and dehulling (van der Poel et al. 1992; Mariscal-Landín, Lebreton, and Sève 2002). Overall, the prevalence of low-tannin genotype of faba bean production today gives promising future for faba bean to be used in monogastrics diets.

Use in poultry

When legume seeds are applied to poultry feed, anti-nutritional factors are always the major concern. The negative effects of condense tannin and vicine and convicine (VC) on energy utilization are additive, and only condense tannin affects total tract protein digestibility (Vilariño et al. 2009). In addition, VC in faba bean seeds will lead to reduced egg sizes and total production when fed to laying hens. As a result, when used in poultry diets, faba bean cultivars with low tannin and VC contents should be considered. Fortunately, the progress made in breeding provides us with choices of faba bean with low tannin and VC contents, which makes application of faba bean in poultry feed possible. In the study of Perez-Maldonado, Mannion, and Farrell (1999), when faba bean was fed to layer hens, egg production was lower for faba bean based diet compared with field peas, chick peas and sweet lupins based diets. Consequent experiment about the optimum inclusion level of legumes for broiler found that faba bean based diet

acquire better growth rate and feed efficiency than sweet lupins and chick peas and optimal inclusion rate for broilers was recommended for 200 g/kg; moreover, steam pelleting treatment of the diets gave better results (Farrell, Perez-Maldonado, and Mannion 1999). Another study was conducted to feed faba bean to broilers in both mashed and pelleted forms and the author found that pelleted faba bean did not impair growth rate and feed efficiency but growth rate and feed efficiency decreased linearly with increasing mashed faba bean (Gous 2011). Application of faba bean to partly replace soybean in organic chicken diets was also tested, and the results show it is inapplicable to use faba bean in organic chickens as nutritional requirements cannot be met (Bosco et al. 2013). Crépon et al. (2010) suggest that the use of faba bean cannot exceed 7% of the diet with high VC content, but when VC content is low, the inclusion rate can be up to 20%. Overall, application of faba bean in poultry diet is suitable when cultivars with low VC and tannin contents are used and faba bean is pelleted.

Use in ruminants

Because of the function of rumen microbiota, condense tannin content in faba bean is not a concern for ruminants, instead it slows down protein digestion and increases protein utilization when condense tannin level is lower than 5% of total DM intake (He et al. 2019). Most of the studies have been focusing on feeding faba bean seeds to ruminants, only limited studies were conducted to test the feeding value of faba bean forage and faba bean hulls. Use of faba bean seeds to substitute soybean meal in ruminants' diet have acquired good results. It is also found that by using faba bean seeds to replace soybean meal, the feed consumption, animal growth and animal products of ruminants can be maintained (Crépon et al. 2010; Cherif et al. 2018; Mendowski et al. 2019). Use of faba bean hulls in the diet is not suggested because of cellulose content is high (50% DM) and most of the protein is rapidly degradable (Minakowski, Skórko-Sajko, and Falkowska 1996). The nutritional value of whole plant faba bean forage wass also tested, but limited studies were conducted to compare faba bean silage with commonly used corn and alfafa silage. The study of Mustafa and Seguin (2003) tested the ensiling ability of faba bean and according to the results, faba silage compared with soybean silage and pea silage had the highest CP content, similar ruminal NDF digestibility and lower CP and DM ruminal digestibility. In addition, McKnight and MacLeod (1977) compared the feeding value of faba bean and grass-legume silages, and similar milk protein, solids contents and milk production were observed, which indicates that whole plant faba bean may be a satisfactory alternative forage.

Faba bean seeds as feed

Most of the application of faba bean in animal science feed have been focused on the use of faba bean seeds as a protein resource. Compared with other raw legume seeds, the solubility and degradability of nitrogen fraction are also higher in faba bean (Sauvant 2004). However, the anti-nutritional factors in the seeds are the major concern. For feed, small seeds cultivars with low tannin contents are mainly used which are cultivars of Snowdrop, Snowbird, Imposa, and Tabasco.

Faba bean whole plant as a forage source

Forages are important feed resources for ruminant. They provide not only energy and protein but also fibers that are crucial in maintaining rumen health. The most commonly used forages in ruminant diets are legumes, corn silage and grasses (Linn and Kuehn 1996). Forages are provided to animals in different forms, which include dry forage (hay), silage, pasture and green-chop. Hay is the traditional forage during the barn feeding season and is made to be easily transported and safely stored. However, producing is influenced by weather conditions and requires more difficult mechanization compared with silage which entails less weather hazard. Pasture, on the other hand, varies in feed quality that depends on plant species, growth stage and available material.

Forages are difficult and expensive to transport because of bulkiness, as a result, alternatives are needed when common used forages are in short (Farminfo 2002). Faba bean has been shown its potential to be a good alternative for forage resource (Sheaffer, Devine, and Jewett 2001). Faba bean crop is a good source of protein and fiber. Moreover, dry matter (DM) and water-soluble carbohydrate (WSC) are usually low in whole-crop faba bean with buffering capacity sometimes high (O'Kiely et al. n.d.). Furthermore, faba bean is resistant to different weather conditions and faba bean forage has been reported to produce 7.8 tons ha^{-1} DM yields with CP concentrations of 18% (Fraser, Fychan, and Jones 2001; Singh et al. 2013). However, attention have concentrated on faba been seed as an optional protein and energy feed resource in the past, limited concern has been paid to the use of faba bean whole plant as a forage resource.

According to Fraser, Fychan, and Jones (2001), different growth stages influence yield and ensilage potential of faba bean silage and the optimum growth for faba bean silage occurred at 14 weeks of growth. Researches have been conducted to ensilage whole plant faba bean, according to the results, both expected lactic acid fermentations and unsatisfactory clostridial can happen during fermentation, hence, additives (or co-ensilage) and proper wilting is needed to maintain a lactic acid dominant fermentation (O'Kiely et al. n.d.; Pursiainen and Tuori 2008; Borreani et al. 2009).

Research to compare rumen degradation kinetics of faba bean with soybean whole crop silage was also conducted, and the results showed that the different chemical composition of these legume silages leads to variation in DM and CP degradability (Mustafa and Seguin 2003).

Faba bean silage was also compared with grass-legume silage in terms of chemical compositions. According to McKnight and MacLeod (1977), faba bean was higher in protein (20.1 vs. 16.17%), lower in crude fiber (25.0 vs. 29.6%), ether extract (1.8 vs. 3.2%), ash (7.1 vs. 8.0%), calcium (0.32 vs. 0.85%) and phosphorus (0.39 vs. 0.43%)

compared with grass-legume silage. In addition, when faba bean and grass-legume silage are fed to cows, similar milk protein, solids contents and milk production are observed, which indicates that whole plant faba bean may be a satisfactory alternative forage.

Traditional evaluation methods for faba bean nutritional characteristics

Chemical profile evaluation

The analysis of feedstuff is to acquire feed chemical compositions for formulating animal diets that can have predictable animal productions from livestock (Van Soest and Robertson 1979). After the establishment of the feed proximate analysis in 1860s by German scientists Hunneberg and Stohman, the feed proximate anlysis has been under continuous revisions and updates and its basic concepts have been utilized in feed analysis until today. Instead of acquiring the content of a single chemical components, feed proximate analysis gives us the contents of groups of chemical components with similar chemical structure and conformation that have specific biological function to animals. According to feed proximate analysis, feed compositions are divided into water, crude protein, crude fat (ether extract), crude fiber, nitrogen-free extract and crude ash. However, this approximate fractioning of feedstuff composition cannot fulfill the needs of knowing more precise compositions of feed, especially in its partitioning of feed fibrous fractions.

According to Van Soest and Robertson (1979), the crude fiber method is of concern for not only ruminants but also monogastrics and human nutrition and it needs replacement with more scientific and accurate methods. Specifically, the contents of cellulose, hemicellulose and lignin that make up the cell wall are actually higher than crude fiber content because from crude fiber method as considerable contents of cellulose, hemicellulose and lignin are dissolved. Consequently, the determination of nitrogen-free extract can be problematic. As a result, the detergent system, which further divide neutral detergent fiber (NDF) into acid detergent fiber (ADF) and acid detergent solubles (ADS) was applied. Moreover, the content of water-soluble carbohydrate and starch are determined today. In addition, the accuracy of the parameter 6.25 used to convert feed nitrogen content into crude protein is affected by the proportion of non-protein nitrogen content in the feed. Hence, the content of non-protein nitrogen in feed is commonly determined especially for ruminants.

Application of Cornell net carbohydrate and protein system for nutrition evaluation

Cornell Net Carbohydrate and Protein System is a mathematical model that predicts animal requirements based on physiological status, production purpose and environmental effects and it evaluates feed nutrients supply with not only chemical composition but also rumen microbiology and degradation kinetics (Fox et al. 2004). The first edition of Cornell Net Carbohydrate and Protein System (CNCPS) came out as a series of four papers in 1992 and 1993 (Russell et al. 1992; Sniffen et al. 1992; O'Connor et al. 1993; Fox et al. 2004). After that, CNCPS has gone through continuous updates and nowadays the commonly used version is CNCPS 6.5 for both practical production and scientific research.

Before the release of CNCPS sub-model, use of the proximate analysis method to evaluate feed quality failed to associate complicated rumen microorganism compositions and competition of nutrients between passage and digestion rate. During that time, the NRC system had some limitations in predicting microbial protein production: (1) considered microbial protein production driven by total digestible nutrient (TDN) instead of available carbohydrate; (2) assumed a constant growth efficiency for microorganisms; (3) did not consider the effect of passage rate to actual feed digestion; (4) did not partition rumen microbial population according to fermentation characteristics and (5) did not integrate N digestion to energy availability (Russell et al. 1992). When the first paper of CNCPS model was released in 1992 (Russell et al. 1992), it put complicated microbial digestion into consideration and proposed a kinetic sub-model that divides microbial ecosystem into structural carbohydrate (SC) fermenter and non-structure carbohydrate (NSC) fermenter; hence, it overcame the shortage of NRC model and provided a useful model for ruminant production. The second paper of the series raised a sub-model that predicted degradation of carbohydrate and protein of feedstuffs and the corresponding partitioning of feedstuff carbohydrate and protein fractions (Sniffen et al. 1992). The third paper of the series predicted the nutrient requirement and feed intake based on different physiological states and production (Fox et al. 1992). The fourth paper of the series predicted the amino acids requirement and absorption for ruminants (O'Connor et al. 1993). Overall, the release of the first four papers set the foundation for the CNCPS model.

Since the first release of the CNCPS model it has gone through continuous improvement; the currently used model CNCPS 6.5 version was released in 2015 and feed library and prediction equations have been refined for more precise prediction (Higgs et al. 2015; Van Amburgh et al. 2015).

Energy value estimation

Energy values of feedstuff are important parameters for evaluating feedstuff quality, especially the quality to promote lactation in dairy cattle and meat production in beef. Unlike other nutrients, it cannot be measured in laboratory, instead, it is predicted by equations derived from regression equations. The prediction equations of National Research Council (NRC) are the one widely accepted as authoritative.

Previous edition of NRC-1989 system had a major drawback in experimentally determine the total digestible nutrient (TDN) value based on feed compositions and assigned TDN values to feedstuff with similar composition. However, the assigned TDN value can be inaccurate. In addition, a constant 8% of deduction was assigned as all cows were

believed to consume at three times maintenance (Council et al. 2001). However, the digestibility of feed is reduced with increasing intake. Therefore, feed energy values should be calculated based on actual feedstuff and its function in the diet. As a result, the latest NRC-2001 model overcame the drawbacks of NRC-1989 model by calculating TDN value based on feed compositions and calculating net energy for lactation (NEl) based on actual intake (Council et al. 2001). The consequent digestible energy (DE) value is then calculated based on its feed categories and actual intake. The corresponding metabolizable energy and net energy are then calculated from DE value at actual intake.

In situ technique for estimation of rumen degradation kinetics

Matching energy supply with N release to maximize microbial protein synthesis and feed efficiency has been recognized for a long time. However, before the invention of an *in situ* technique to study the degradation characteristics of feed in the rumen, the synchronization of N and energy in the rumen could not be fully explored. In 1979, the study of Ørskov and McDonald (1979) proposed an artificial-fiber bag technique to determine the rate of degradation of feed in the rumen using cannulated cattle. Along with the technique is the mathematical model used to evaluate kinetics of feed composition that degraded at different time points in the rumen. After the release of this paper, this technique has been widely used in ruminant research, for example to study the synchronization of N to energy in the rumen (Sinclair et al. 1993).

Three-step in vitro technique for evaluation of intestinal digestibility

With the information of microbial protein synthesis and undegradable protein after ruminal digestion, it is necessary to estimate their contribution to animal production through small intestinal digestion. The commonly used *in vivo* method requires fistulated animals thus is labor-intensive and costly and involves animal variance (Stern, Bach, and Calsamiglia 1997). In addition, the lack of empirical data about intestinal protein digestibility was recognized (Stern, Bach, and Calsamiglia 1997). As a result, to overcome the disadvantages of the *in vivo* method and to fill in the gap of protein intestinal digestibility, several methods (bioassays; *in situ* mobile-bag technique and *in vitro* technique) have been utilized in estimation of intestinal protein digestibility; among them the three-step *in vitro* method is of commonly use. Three-step *in vitro* method was firstly proposed by Calsamiglia and Stern (1995) in 1995, and it has gone through revision in 2006 by utilization of a batch incubator (Daisy II) to save the labor and cost (Gargallo, Calsamiglia, and Ferret 2006). Nowadays, three-step *in vitro* method as well as daisy II batch incubator are commonly used in intestinal digestibility estimation.

Prediction of truly digestible protein supply to the small intestine and feed milk value

DVE/OEB system

One of the most important drive of production ability of ruminants is so called metabolizable protein which consists of undegradable feed protein; rumen synthesized microbial protein and endogenous protein. To evaluate the metabolizable characteristics of the feed several models have been utilized namely NRC model, the Dutch DVE/OEB system and the France PDI system. Among them, DVE/OEB system and NRC system are commonly used and undergo continuous revision and update. The DVE/OEB system was firstly proposed in 1991 to replace the digestible crude protein system (DCP) to make the most efficient use of nitrogen (Tamminga et al. 1994).

According to Theodoridou and Yu (2013b), the protein supply to the small intestine of dairy cows of a feed is estimated based on DVE/OEB system which constitute of two parts, the DVE and the OEB value respectively. The DVE value is constituted of digestible feed protein, microbial protein, and an endogenous protein loss correction while the equation is described as: $DVE = AMCP^{DVE} + ARUP^{DVE} - ENDP$, where $AMCP^{DVE}$ is the microbial crude protein that is absorbable; $ARUP^{DVE}$ is the ruminally undegradable feed protein and ENDP is used for correcting the endogenous protein lost during the digestion process. The OEB value is described as equation $OEB = MCP_{RDP}{}^{DVE}\text{-}MCP_{FOM}$. $MCP_{RDP}{}^{DVE}$ is the potential MCP synthesis based on RDP, MCP_{FOM} is potential MCP synthesis using energy from anaerobic fermentation. The $MCP_{RDP}{}^{DVE}$ is calculated as equation $MCP_{RDP}{}^{DVE} = CP \times [1 - (1.11 \times RUP\ (\%\ CP)/100)]$.

NRC-2001 model

Similar with DVE/OEB system, the metabolizable protein supply in NRC-2001 model has the same sources. However, different from DVE/OEB system, the endogenous protein is contributed to the metabolizable protein. According to NRC-2001 (2001), the calculation is given as below. When RDP exceeds 1.18 × TDN-predicted MCP (MCP_{TDN}), MCP_{TDN} (potential ruminally synthesized microbial CP) will be calculated as MCP_{TDN} (g/kg of DM) $= 0.13 \times$ TDN (discounted). When RDP is less than $1.18 \times$ TDN-predicted MCP (MCP_{TDN}), then MCP will be calculated as 0.85 of RDP ($MCP_{RDP}{}^{NRC}$); truly absorbed MCP ($AMCP^{NRC}$) will be calculated as $AMCP^{NRC}$ (g/kg of DM) $= 0.80 \times 0.80 \times MCP_{TDN}$, where true protein and digestibility of ruminally synthesized microbial CP are both defined as 800 g/kg in NRC-2001; $ARUP^{NRC}$ (truly absorbed rumen-undegraded protein in the small intestine) will be calculated as $ARUP^{NRC} = RUP^{NRC} \times$ dRUP (intestinal digestibility of rumen undegraded protein); rumen endogenous protein in the small intestine (ECP^{NRC}) will be calculated as ECP (g/kg of DM) $= 6.25 \times 1.9 \times$ DM; truly absorbed rumen endogenous protein in the small intestine (AECP) value will be calculated as AECP (g/kg of DM) $= 0.50 \times 0.80 \times$ ECP; total metabolizable protein (MP) will be calculated as MP (g/kg

of DM) $=ARUP^{NRC} + AMCP^{NRC} + AECP$; the degraded protein balance (DPB^{NRC}), which describes the difference between potential microbial protein synthesize based on energy RDP, will be calculated as DPB^{NRC} (g/kg of DM) $= RDP^{NRC} - 1.18 \times MCP_{TDN}$.

Contribution of synchrotron-based and globar-sourced mid-infrared (Mid-IR) spectroscopy techniques to evaluate faba bean structural and molecular property

Mid-infrared spectroscopy and Fourier transform infrared spectroscopy (FTIR)

Infrared spectroscopy is the applicable analytical technique emerged from the 1940s and nowadays it has become a powerful tool commonly used in identifying organic compounds, characterizing polymers and studying biological molecules (Stuart 2015). In addition, it is a nondestructive and rapid analytical technique which can be applied in samples of different states such as lipids, powders, solution, films and gases (Stuart 2015). According to wavenumber range, an infrared spectroscopy can be divided into three main regions: the far-infrared region (wave number less than 400 cm^{-1}), the mid-infrared region (wavenumber between 4000 and 400 cm^{-1}) and the near-infrared region (wavenumber between 13000 and 4000 cm^{-1}). Both near-infrared (NIR) and mid-infrared (MIR) are commonly used in food and feed analysis to obtain both quantitively and qualitative relationships (Li-Chan, Chalmers, and Griffiths 2010). Far-infrared instead is mainly used for the investigation of inorganic substances (Prati et al. 2011). While NIR informs on harmonic and overtone absorptions, MIR acquires the spectral based on fundamental molecular vibration, which explains the reason why MIR gives better insight molecular bonds (Nikolic 2011). In other words, spectra recorded from near-infrared (NIR) range are "sensitive to multitude of compounds and molecular interactions" and NIR is superior to MIR in quantitively predicting nutrient compositions in grains (Hell et al. 2016; Shi and Yu 2017). However, NIR spectra fail to identify more complex and similar structures, which could be achieved with mid-infrared spectroscopy (Hell et al. 2016). In general, MIR is more commonly used in qualitatively identify and verify chemical compounds while NIR is more commonly used to quantitively predict chemical compositions in the sample (van de Voort 1992).

The introduction of Fourier transform infrared spectroscopy (FTIR) spectrometers has significantly improved the utilization of mid infrared spectroscopy. Compared with conventional IR spectroscopy which utilizes individual wavelength, FTIR spectroscopy makes use of a complete source spectrum and thus having several advantages over dispersive type instruments, such as higher signal-to-noise ratio, less scan time and superior scan accuracy (van de Voort 1992). The basic idea of FTIR spectroscopy is to obtain an interferogram by interference of two beams and using a mathematical method called Fourier transformation to yield the spectrum (Stuart 2015). The core component of FTIR spectrometer is the interferometer which consists of two perpendicular plane mirrors, one of which can travel perpendicularly to the other, a beamsplitter bisects the plane of the two mirrors, a radiation source and a detector (Stuart 2015).

Basic principles of IR spectroscopy

The total energy of a molecule consists of translational energy, rotational energy, vibrational energy and electronic energy. When infrared radiation is processed to the organic molecule, the molecular equilibrium stage is broken, which promotes the rotational and vibrational energy of the molecule and leads to the unique absorption of infrared radiation because of the unique chemical bonds and functional groups of organic matters (Colthup 2012). Within the mid-infrared region, several spectral regions are mainly identified which are X-H stretching region (4000–2500 cm^{-1}), triple bond region (2500–2000 cm^{-1}), double bond region (2000–1500 cm^{-1}) and the fingerprint region (1500 to 600 cm^{-1}) (Stuart 2015). The fundamental vibrations in X–H stretching region are generally aroused by the stretch between the hydrogen atom and atoms like carbohydrate, oxygen and nitrogen. Specifically, N–H, O–H and C–H stretching occur in 3700–3600, 3400–3300 and 3000-2850 cm^{-1}, respectively (Barbara H Stuart 2004). Triple bond stretching absorptions are due to C≡C and C≡N but sometimes X-H stretching may also fall in this region, where X is atom with bigger atomic mass like silicon and phosphorus. In double bond region the vibration is generally caused by C=C, C=O and C=N stretching with the carbonyl stretching one of the most noticeable absorption in an infrared spectrum. The chemical composition and structural information can be acquired by identifying the unique absorption of IR radiation. Unlike in other regions where each band can be assigned to specific characteristics of a molecule, in the fingerprint region, the vibrations are extremely variable and are even unidentical for similar molecules (Stuart 2015). As a result, the spectrum in the fingerprint region is unique and can be considered as the fingerprint of the molecule.

Application of MIR technique in nutritional analysis

Besides the amino acids conformation, proteins are exquisitely assembled to its unique three-dimensional structure (secondary structure) to fulfill its functions, and the secondary structure of protein includes mostly α-helix, β-sheet, with small proportion of β-turn and random coils. Since the 1950s, the correlation between the secondary structure of protein and the frequency of infrared spectroscopy absorption in so-called amide I and amide II regions has been found; specifically, the vibration in amide I region was found to be related to mainly C = O stretching and partly C–N stretching, while N–H bending and C–N stretching contribute to the vibration in amide II region, in other words, the frequency of amide I and amide II absorptions is related to any hydrogen bonds involved with amide C = O

and N–H groups (Jackson and Mantsch 1995). As different proteins have unique secondary structure which can be reflected in the distinctive hydrogen bonding pattern between C = O and N–H groups, thus the infrared absorptions in amide I and amide II region can be utilized to determine distinguished secondary structure of protein (Jackson and Mantsch 1995).

In feed industry, the feeding value of protein in feedstuff is mainly considered from the perspective of amino acids composition and protein digestibility without taking secondary structure of protein into consideration. However, the feeding value of protein is also influenced by the structure of protein in digestive behavior, nutritive quality and digestibility in animals, as the secondary structure of protein is highly associated with the specific susceptibility to the enzymatic hydrolysis (Yu 2005a, 2007). According to Yu (2004b), feathers which are commonly used as a source of protein supplement are very high in crude protein content, but the protein digestibility is very low; and the low protein digestibility is associated with its secondary structure as feather protein has a high β-sheet ratio compared with other grains. In addition, the protein nutritional profiles of feed are found to be closely correlated to its spectral profiles (Li, Zhang, and Yu 2016; Lei et al. 2019). As a result, FTIR, which is capable of detecting the secondary structure of feed sample is a valuable tool in assessing the feed value in regard with its protein secondary structure (Yu et al. 2004b; Yu 2005a, 2005b).

Plant cell wall structure is important in determining the nutritional value of feed to ruminant. Different polysaccharides associated with hydrogen bond to form three types of structurally independent polymer networks: cellulose microfibrils connecting with non-cellulosic polysaccharides (for example phenolic heteropolymer lignin), gel-like matrix of pectin linkage with other polysaccharides and their cross-linkage with structural protein (Pettolino et al. 2012). Polysaccharides linkage between each other and linkage between polysaccharide and protein and lignin can all be detected by FTIR and contributed to unique spectra. In addition, the change of plant cell wall structure by processing or biohydrogenation can also be reflected in its change of chemical bonds and can be easily detected by FTIR.

Feed processing is commonly used in feed industry to reduce anti-nutritional factors, to increase feed efficiency and digestibility. The accompanied protein structure alteration during the feed processing was found to affect the digestive behavior, nutrient utilization and availability of protein, but traditional wet chemistry analysis methods fail to unveil the internal relationship between the change of protein structure feature and the change in nutritional value. FTIR is capable of detecting the change in protein without destroying the protein structure, thus FTIR is proved to be able to give better insight in understanding the intrinsic protein molecular structural changes during processing (Yu 2007; Yu and Nuez-Ortín 2010; Theodoridou and Yu 2013a, 2013c; Yu et al. 2015). In recent years, the by-products of ethanol industry, distillers dried grains with solubles (DDGS) have entered the feed industry as a raw material. Yu and Nuez-Ortín (2010) compared different types of DDGS, such as wheat DDGS, maize DDGS and blend DDGS (wheat: maize = 70:30) regarding the processing related change in IR spectroscopy (α-helix, β-sheet, amide I and amide II and their ratios) and their protein nutritive values (rumen protein digestibility, intestinal protein digestibility and truly absorbable protein in the small intestine), that the changes of spectral profiles with bioethanol processing were highly associated with its protein nutritive values, and suggested the protein structure features should be considered in evaluating the protein value for the feedstuff.

In addition to detecting the change of protein secondary structure (α-helix and β-sheet) of feed during processing and differentiating feed protein quality according to protein spectral profiles, FTIR has also been proved to be able to detect changes of molecular structure during gene modification. In recent years, significant progress has been made in gene modification to improve forage quality for sustainable agriculture development and to create tremendous economic benefits (Wang and Ge 2006). Gene modification of forage is mainly focusing on manipulating gene expression in important metabolic pathway to acquire better nutritive value, productivity and improved agronomic characters. However, the change of molecular structure has also been found to happen during gene modification and such changes are related to the nutritive value of forage, and FTIR is capable of detecting such change (Jonker et al. 2012; Lei, Hannoufa, and Yu 2017, Lei et al. 2018, Lei et al. 2019). According to Lei et al. (2019), the silencing of HB12 and TT8 genes in alfalfa aiming at reducing tannin content in alfalfa, decreased the protein degradation and metabolic profiles, and the changes in nutritional and digestive characteristic are closely correlated to their spectral features. In addition, Badhan et al. (2014) successfully utilized FTIR to differentiate the alterations in cell wall architecture generated by gene modification.

In addition to amide I and amide II regions, other spectral regions have also been found to be highly correlated to nutritional characteristics and structural characteristics of feed. Just like protein, carbohydrate and lipid also have their unique molecular and chemical features, which are in relation to their structural characteristics; specifically, lipid contains C = O carbonyl as well as CH2 and CH3 functional groups, while the unique structural chemical-structural features of carbohydrates are OH and CO bonds (Yu et al. 2004a). According to published papers (Himmelsbach, Khalili, and Akin 1998; Wetzel et al. 1998, Wetzel, Srivarin, and Finney 2003; Yu 2012), the spectral regions related to carbohydrate and lipid have been identified. As for carbohydrate related spectral profiles, spectral region between 1485 and 1188 cm^{-1} is mainly associated with hemi-cellulosic and cellulosic compounds; spectral region between 1292 and 1198 cm^{-1} is mainly associated with cellulosic compounds; spectral region between 1187 and 950 cm^{-1} is associated with total carbohydrate (Yu 2012). As for lipid related spectral profiles, there are four peaks at 2955, 2873, 2922 and 2843 cm^{-1}, which are attributed to CH2 and CH3 asymmetric and symmetric stretching vibrations, and lipid carbonyl

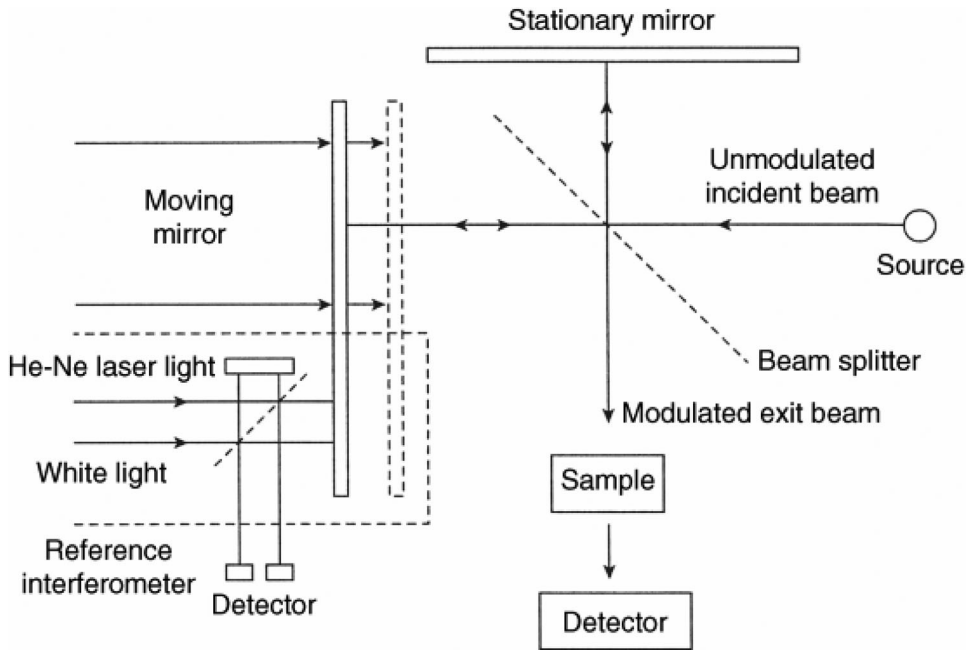

Figure 1. Michelson Fourier Transform Infrared Spectrometer (FTIR) interferometer. Adapted from Stuart (2004).

C = O ester is associated with spectral region between 1774 and 1771 cm^{-1} (Morent et al. 2008; Abeysekara et al. 2012). Unlike in fingerprint region where vibration induced by infrared radiation is unique and can be considered as the fingerprint of the molecule, in carbohydrate and lipid related spectral regions, each band in the region is associated with specific characteristics of a molecule, in other words, the spectral profiles in carbohydrate and lipid related regions reflect more information in intermolecular reaction rather than molecular structure. According to published papers (Abeysekara et al. 2012; Xin, Falk, and Yu 2013, Xin et al. 2014; Xin and Yu 2014), both the carbohydrate and lipid related spectral features are correlated to nutrient values to some extent, and may be used to predict nutritional supply to animals (Xin, Falk, and Yu 2013). As a result, FTIR is utilized to reveal the association between internal molecular structure and nutritional and digestive characteristics of feeds, as well as the possible alteration of structure during processing (Rodríguez-Espinosa et al. 2020). However, researchers fail to interpret more information in the spectra, thus cannot apply the principle to a broader scale. To be specific, the correlation and regression relationships between spectral features and chemical and digestive behavior of feed acquired from one treatment cannot be applied to another one, which narrows its application in the industry as a routine evaluation.

Although NIRS has been approved by the Association of Official Analytical Chemists (AOAC) in the quantitively determination of crude protein, moisture and acid detergent fiber content in feed (Undersander 2006), it fails to detect more subtle difference between molecules. The technical improvement made in Fourier transform instruments and sample preparation in recently years enables MIRS to successfully detect more complex molecules in feed (Sherazi et al. 2007; Allison et al. 2009b, 2009a; Nikolic 2011). According to the study by Sherazi et al. (2007), FTIR successfully distinguished free fatty acids from other lipids and was proved to be an efficient and repeatable method to determine free fatty acids content in poultry feed lipid extracts. In addition, Allison et al. (2009b, 2009a) used FTIR with partial least square regression to predict lignin and hydroxycinnamic acids, nitrogen and alkali index in grasses. As a result, FTIR shows its possibility to be utilized as a quick, nondestructive and environment-friendly method in quantitively predicting feed composition of some molecules with similar structure which cannot be predicted with NIR and chemical analysis.

Using synchrotron-based FTIR microspectroscopy (SR-IMS) to reveal intrinsic molecular structures in feed

While conventional IR spectroscopy can only compares sample structural chemical features generally, synchrotron based FTIR microspectroscopy gives plant structural chemical information within cellular dimensions (Yu et al. 2004a). The basic principle of SR-IMS is similar to IR spectroscopy as they both use radiation within IR region, however, SR-IMS utilize synchrotron beam light as light source which is 100-1000 times brighter than a conventional globar source, thus providing higher accuracy and precision with resolutions as fine as 3-10 µm and high signal: noise ratio (Yu 2004; Yu et al. 2004a). Although SR-IMS is a newly developed bioanalytical tool with promising potential, its application in agriculture has not been fully explored. Figure 3 gives synchrotron-based Fourier transform infrared. Microspectroscopy spectrum and its 2nd derivative andFourier Self Deconvolution spectra for triticale in the amideI and amide II regions.

One example of utilizing SR-IMS in agriculture is illustrated by Yu et al. (2004a), spectral related to Amide I region was applied to a sample, according to the principle illustrated in the beginning of the article, amide I region is

related to protein, as a result, the uneven distribution of protein in the plane tissues can be informed by chemical image, and obvious higher protein content in sub-endosperm is observed, with minimal protein content in pericarp. The example is used here to give a brief impression of what SR-IMS can achieve.

Detailed explanation of present application of SR-IMS to animal nutrition and feed science was reviewed by Yu (2004), according to which the applications are: comparing the chemical-structural features of feed tissues according to spectroscopic characteristics in relation to its chemical composition; and may further utilizing the ultrastructural-chemical makeup and density to predict feed nutritive values for breeding; in addition, the chemical-structural differences in endosperm tissue between two types of barley could be related to rumen degradation characteristics. However, how can we combine the SR-IMS technique with *in situ* and *in vitro* studies to further research the influence of nutrient distribution on the digestive behavior of animals deserve fully consideration. Specifically, how the distribution of carbohydrate (both structural and nonstructural carbohydrate) and protein in the feed is related to microbial digestion behavior in the rumen, and furthermore to animal performance deserve further research.

Disclosure statement

No potential conflict of interest was reported by the authors.

Acknowledgements

The Ministry of Agriculture Strategic Research Chair (PY) Programs are supported by various funding agencies and industry and producer organizations: the Natural Sciences and Engineering Research Council of Canada (NSERC Grants), the Saskatchewan Pulse Growers (SPG), the Agriculture Strategic Research Chair Program Fund, The Prairie Oat Growers Association (POGA), the Agricultural Development Fund (ADF), the SaskMilk, the SaskCanola, the Saskatchewan Forage Network (SNK), the Western Grain Research Foundation (WGRF) etc. The Misiwêskamik International Postdoctoral Fellowship (XF) from the University of Saskatchewan is acknowledged. The National Synchrotron Light Source in BrookhavenNational Laboratory (NSLS-BNL, New York, USA),Advanced Light Source in Berkeley National Laboratory (ALS-BNL) are supported by the U.S. Department ofEnergy. Canadian Light Source Inc. (CLS) at University ofSaskatchewan (Saskatoon, Canada) is supported by variousCanadian federal and provincial funds. The authors aregrateful to Na Liu and Chithra Karunakaran for helpful synchrotrondata collection at CLS, Lisa Miller (NSLS-BNL,New York) for synchrotron beamtime arrangement at ALSand NSLS, valuable discussion and/or collaborations, andRandy Smith (NSLS-BNL, New York) and Hans Bechtel (ALS, Berkeley) for helpful synchrotron data collection atALS and NSLS

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