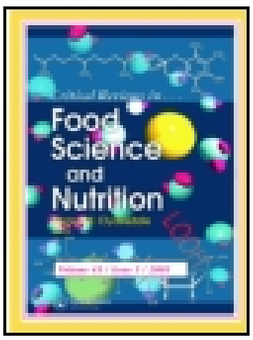

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Review of analytical methods for measurement of oat proteins: The need for standardized methods

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ABSTRACT
This review summarizes the analytical methods that have been developed for quantification and characterization of oat proteins. These include sampling, sample preparation, extraction, quantification, separation, detection, and characterization of oat proteins. The review also provides a comparison of different methods for the determination of protein fraction of oat and the efficiency thereof. We conclude that there is a need for further validation of existing data or methods and for a standard methodology to quantify oat proteins.

KEYWORDS
Extraction; separation; detection; characterization; efficiency; harmony

Table of Contents

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1. Introduction

Historically, oat (Avena) has been used as an important food component for cattle and for humans because it contains ingredients that satisfy nutritional needs. Oats are primarily used as an energy feed because their gross energy is superior to that of any other cereal due to their high fat (5 percent), unsaturated fatty acids, unique galacto lipids and high level of globular proteins with a favorable amino acid profile (Shewry and Halford 2002a, Peterson 1978, Zarkadas, Yu, and Burrows 1995, Aaman and Graham 1987, Maruyama et al. 1975, Paton 1977, Brown and Craddock 1972). Recently, acknowledged health benefits and nutritional factors have led to an increased desire to use oats as an ingredient in new food products for human nutrition. Moreover, the protein content of oats is usually 9–15 percent (Maruyama et al. 1975) and can be as high as 20 percent (McMullen 1991), which is higher than in other commercial cereal grains such as corn, barley, wheat, and sorghum. However, the protein content in oats is highly variable. Genetic and environmental factors such as different fertility levels are known to affect the protein levels in different oat cultivars (Peterson and Smith 1976, Völker 1975). Moreover, a lack of standard extraction choice and concentration of ions, temperature, and pH and analytical methods may contribute to variation in the measured protein content. Therefore, it is difficult to compare different cultivars, especially for globulin protein, the major protein component of oats (Peterson 1978, Webster 2011).

A wide range of analytical methods have been developed to analyze and quantify oat protein since the 1970s. Several scientists characterized nutrition regimes of oats during the 1970s and 1990s (Brinegar and Peterson 1982, Donhowe and Peterson 1983, Maruyama et al. 1975, Ma and Harwalkar 1984, Åman 1987, Wu 1973). Nevertheless, quantitative and qualitative determination of total proteins have generally been unsuccessful (Lasztity 1995, Peterson, Brinegar, and Webster 1986) because of the lack of a standard characterization method (Shewry and Halford 2002b). Variation and improvements in the extraction, quantification, and classification of different protein fractions of these methods are still ongoing worldwide. Considering the importance of cereals as sources of protein, both for humans and animals, analysis of protein quantity and quality is inevitable, which means that a standardized and widely accepted extraction and analytical method to understand the nature and functionality of cereal proteins is essential. To best of our knowledge, no standardized extraction and analytical method for quantitative analysis of oat proteins has yet been proposed, which makes it difficult to compare data from different laboratories. Therefore, the current review aims to summarize and compare the available extraction and analytical methods for protein in oats.

2. Methodology

We performed literature review using online databases such as Google Scholar, PubMed, Web of Science, Scopus, MEDLINES, Wiley Online Library, and Science Direct, for keywords and phrases such as oat proteins fractions, oats and amino acids, oat globulin, albumin, soluble proteins, protein fractions, analytical methods, chromatography, spectroscopy, and molecular methods from 1970 to 2015. We found more than 2000 articles. The exclusion criteria included (1) irrelevance of the subject, (2) lack of information (3) papers that did not detail any chemical or analytical methods for oat protein analysis. We ended up selecting and reviewing 137 articles from various countries, comprising original research articles, book chapters, and previous relevant review papers. We also cited 4 old papers (published before 1970) in this review because of their relevance to the current review for comparing the data.

3. Results

In total, we reviewed 137 articles published between 1970 and 2015. The details of the most used technique or most relevant data (73) found in recent literature are displayed in Table 3.

3.1. Sampling and storage

The sampling method and the site at which the samples were obtained are the two important factors that a researcher must consider before the experimental analysis. In cereals, especially oats, sampling location has an effect on the concentrations of total protein and protein fractions (Peterson and Smith 1976, Wu et al. 1972, Matlashewski et al. 1982, Webster 2011,

Forsberg et al. 1992, Forsberg, Youngs, and Shands 1974, Peterson et al. 2005). In the 137 articles referred to here, the authors defined the sample location as the respective field from which they obtained the groat/seed or oat flour and eventually stored it at room temperature. To the best of our knowledge, no studies have evaluated the effect of storage temperature and storage time on the protein content in oats. Nevertheless, studies has been found that storage of undeveloped harvested seeds or caryopses removed from the florets in the freezer or in liquid nitrogen for the polysoms extraction had no deleterious effects on their amino acid profile. Also, no differences in protein expression in grains were noticed between *in vitro* and *in vivo* protein abundance analysis using whole spikelets or detached seeds stored in liquid nitrogen (Luthe and Peterson 1977, Matlashewski et al. 1982, Walburg and Larkins 1983).

3.2. Sample pretreatment

Sample pretreatment and processing are critical factors for most of the identification and characterization of cereal protein fractions. Whenever proteins are useful for nutritionally important products, the naturally associated insoluble components of oils associated with the protein should be removed prior to processing (Potter and Bixby 2000, Ekstrand et al. 1993). In oats, the hull must be removed or dehulled before the grain is processed into a finished product. Dehulling can done using an impact dehulling machine known as a huller, or manually for experimental purposes that require only a couple of grams of oat flour. A few studies have evaluated the impact of hulling process on damage or nutritional changes of oat groats (Osborne and Anderssen 2003, Doehlert and McMullen 2000, Engleson and Fulcher 2002, Doehlert, McMullen, and Baumann 1999, Doehlert and Wiessenborn 2007). When the hull is removed through milling, 4–5 percent increase in groat percentage has noticed. The flour obtained from dehulled seeds showed good functional properties compared to oat flour made from seeds with the non-digestible oat hull (Doehlert, McMullen, and Riveland 2010). The protein content of hulls ranges from 2.2–4 percent (Wu et al. 1977, Welch, Hayward, and Jones 1983). The next step is to grind the dehulled oat into fine powder; this is usually done with a pine mill or hammer mill (David 2011). Researchers noticed the differences in concentration of protein of oat flour prepared after different dehulling procedures (Doehlert and Moore 1997). A maximum yield of protein (83–88 percent of total protein) was obtained from oat flour milled by a Bühler mill compared to hammer and pine mill (Wu et al. 1972).

The results of several studies indicate that the nature of milling, such as wet or dry milling, can lead to a considerable improvement in the yield of protein concentrates. The nature of milling could probably affect nitrogen solubility as well as functional properties (Cluskey et al. 1973, Wu et al. 1973, Wu 1973). Air classifications methods, together with dry milling, gave protein yield ranges from 4–88 percent. In addition, density separation using Freon- hexane mixtures gave 70 percent protein, which represented 40 percent of the protein in the flour. Wet milling followed by extraction of protein concentrates at acidic and basic pH produced a higher yield of protein concentrates with good functional properties than the dry milling (Cluskey et al. 1973).

The defatting step is required to remove the lipids and other fats in the oat flour, thereby extending its storage time and reducing interference from the fats during extraction procedures. Defatting is usually done via hexane, acetone, and butanol or soxhlet extraction with hexane (Wu et al. 1977, Runyon et al. 2013, Wu et al. 1973, Ma and Harwalkar 1984, Pernollet, Kim, and Mosse 1982, Zhao, Mine, and Ma 2004). Wu et al. (1977) noticed that the butanol-defatted flour gave a slightly higher yield of protein isolate and total protein content than hexane-defatted flour. Negligible amounts of nitrogen solubility differences were also noticed between hexane- and butanol-defatted flours, and the defatted oat flour gave a slightly higher protein content than the un-defatted oat flour (Runyon et al. 2015, Wu et al. 1977, Ma and Harwalkar 1988).

3.3. Total and soluble oat protein quantification

Quantification of total protein content in a sample is critical for any protein analysis. The earliest method used to quantify oat protein was the Kjeldahl Method. Though modified versions of Kjeldahl methods, such as Microkjeldahl, Kjel Foss, or Kjel-Foss automatic protein analyzer have been reported, recovery was poor (below 50 percent) (Wu 1973, Luthe 1987, Luthe and Peterson 1977, Kim, Charbonnier, and Mosse 1978, Pernollet, Kim, and Mosse 1982, Ma 1984, Ma and Khanzada 1987b, Nnanna and Gupta 1996, Ma and Wood 1987b, Redaelli, Sgrulletta, and Stefanis 2003, Ma, Harwalkar, and Paquet 1990). Lowry's method has also been used for total protein quantification of oats (Ma and Harwalkar 1984). Currently, the other most commonly used methods are using the principle of dye – binding assay (Adeli and Altosaar 1983), lab-on-a-chip analysis (Klose and Arendt 2012), the Dummas method, AF4 (Asymmetrical flow field flow fractionation) (Runyon et al. 2013, Runyon et al. 2015), spectrophotometry (Ma and Harwalkar 1988), and the BCA method (Gonzalez, Koren'Kov, and Wagner 1999). However, in order to obtain an accurate value of the protein content of the grain, the generally accepted nitrogen-to-protein (N: P) conversion factor must be used. Because the nitrogen: protein ratio varies between food stuffs, the final protein value must be accurately evaluated when expressing nitrogen as protein (Mariotti, Tome, and Mirand 2008). For oats, the N: P conversion factor is 5.36 $\pm$ 0.05. This factor has been determined based on the upper and lower limits obtained for the percentage of nitrogen in the seed (Mosse 1990, Yamaguchi 1992, Tkachuk 1969, Smith).

3.4. Extraction of protein

3.4.1. Extractions in buffer

The earliest method for classification of plant proteins was developed by US biochemist T.B. Osborne (Osborne 1907). A series of extraction buffers were used to extract, quantify, and classify cereal storage proteins. Osborne identified four solubility fractions in oats: salt-soluble globulin, water-soluble albumin fraction, alcohol-soluble prolamins, and glutelins that is soluble in dilute acids or alkalis. Process variation and continuous improvements of this method are still being used today (Wu et al. 1972, Völker 1975, Peterson and Smith 1976, Wieser and Belitz 1989, Ma and Harwalkar 1984, Bean, Bietz, and

Lookhart 1998, Robert, Nozzolillo, and Altosaar 1985). Later, Peterson and Smith characterized proteins from different oat varieties using Osborne's fractionation and calculated that albumins (water-soluble), globulins (salt-soluble), prolamins, and glutelins represented 14–19 percent, 52–53 percent, 7–9 percent, and 21–27 percent of total protein, respectively (Peterson and Smith 1976). The advantages of Osborne's fractionation are rapidity and simplicity; however, the traditional Osborne method could not achieve quantitative extraction of unique group of polypeptides, as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), for each fraction. Thus, although only a few studies directly compared the solubility and extractability of oat protein using different buffers and different extraction procedures, it has been observed that the proportion of oat protein fractions depends on buffer concentration, pH, temperature, and growing environment (Peterson 1978, Völker 1975). Many discrepancies were found in earlier records regarding the proportion of oat solubility fractions, especially globulin and glutelins. Peterson and Smith found that 45–50 percent of total nitrogen was endowed with globulin in several oat cultivars. Several German scientists noted that the glutelins make up the predominant fraction of and that the concentration of globulin was only 12–19 percent of total protein (Völker 1975, Wieser, Seilmeier, and Belitz 1980a).

When several extractions were carried out with typical Osborne fractionation, comparison in ana sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed that globulin makes up the major fraction of protein. The SDS-PAGE found no major polypeptide for glutelins (Robert et al. 1983). Precipitation of all protein using salt solution and further extraction of un-extracted protein/polypeptide with SDS/mercapto ethanol further confirmed that globulin makes up the highest portion of oat protein and the glutelins probably do not act as a major storage protein in oats (Robert, Nozzolillo, and Altosaar 1985, Luthe and Peterson 1977, Walburg and Larkins 1983, Brinegar and Peterson 1982). Similarly, nitrogen analysis of dry-milled protein fractions separated using sodium chloride and sodium acetate claimed a high amount of globulin in their extraction (Wu et al. 1972). Ma and Harwalkar (1984) also showed that 0.5M $CaCl_2$ produced a higher yield of globulins (Ma and Harwalkar 1984); this emphasizes the importance of a most suitable solvent for the extraction of all protein fractions from oats.

3.4.2. pH and ionic concentration

The solubility, emulsifying properties, hydration capacity, and organoleptic properties of oat proteins depend upon the pH at which the protein concentrate was obtained. Ma et al. (1983) studied the solubility of cv. *Hinoat* and cv. *Sentinel* oats as a function of pH and obtained a bell-shaped curve with the lowest protein solubility at a pH of 5–6, with 15 percent of the nitrogen soluble at pH 5 (Ma and Khanzada 1987b). The unmodified and deamidated oat protein isolates from *cv. Garland* also achieved a bell-shaped curve under varying pH (pH 4– 8). An increase in solubility was found with more acidic or alkaline conditions. The decreases in nitrogen solubility with increasing ionic strength were also observed with oat proteins (Wu 1973).

A considerable amount of increase in protein concentrate was noticed at a pH 5.7 from basic pH for both wet- and dry-milled oat flour (Wu et al. 1973). This change in solubility could be due to a decrease in molecular size and an increase in net charge on protein molecules that can promote a strong protein water interaction and hence an increase in solubility (Kinsella and Shetty 1979). In addition, a change in the solubility of defatted *Garland* groat protein was noticed with NaCl under different ionic strength and varying pH (6–10) levels. Solubility at pH 12 was 83 percent in 0.1 mol/ L NaCl that was decreased to 37 percent at pH 2.2 in 0.1 mol/ L NaCl. However, in 1 mol/ L NaCl at pH 2.19, only 9 percent was found soluble. The strong influence of pH in protein polypeptide shift was noticed in FT/Raman spectroscopy (Ma, Rout, and Mock 2001). There was a shift from native structure due to a stretching of amide bands during protein denaturation that resulted in a transition from a beta sheet structure near neutral pH (pH 7) to a random coil structure at extremely acidic (pH 3) and alkaline pH (pH 9–11) (Ma, Rout, and Mock 2001).

3.4.3. Protein concentrate

Wet- and dry-milled processes for obtaining protein concentrates or isolates from groats showed different amounts of protein depending on the extraction buffer and pH. An alkaline extract above pH 9 gave 59–89 percent of protein with good N solubility and functional properties (Cluskey et al. 1973). An air classification method gave 4–88 percent, with a good amino acid composition, which was 14–16 percent of the total protein of the flour. Density gradient separation using Freon–Hexane mixtures obtained 70 percent, which is 40 percent of the protein in the flour. The pH at which extraction was performed with both wet- and dry-milled oat flour has an influence, not only on the final amount, but also on the functional and organoleptic properties of protein concentrates. Isoelectric precipitation of alkaline extracts and dialysis or dilution of salt solution extract gave more than 90 percent of protein. Oat protein has generally suffered low solubility and emulsion properties at pH 4–7 (Wu et al. 1973).

3.5. Separation techniques

A number of studies have been devoted to the separation, physicochemical characterization, and determinations of oat protein fractions, either jointly or separately. Analytical ultracentrifugation, electrophoresis such as SDS gel electrophoresis, isoelectric focusing (IEF), capillary zone electrophoresis for separations of protein fractions, and several chromatographic methods for measuring protein components are all listed below. In addition, a number of immunological methods and enzymes are practiced.

We used several types of detectors in the analysis, with our main focus on ultraviolet detection (UV), fluorescence detection (FD), spectrophotometry (MS), and multiangle light scattering (MALS) detections.

3.5.1. AUC (analytical ultracentrifugation)

Analytical ultracentrifugation is a powerful method for the quantitative analysis of macromolecules in solution. Using ultracentrifugation, Peterson (1978) characterize globulin and proposed a hexametric nature of oat globulin based on the

sedimentation co-efficient. Globulin showed a sedimentation constant of 12.1, and a molecular weight of 322,000 Daltons (Peterson 1978). In SDS-PAGE, two polypeptides with molecular weight 21,700 α (alpha) and 31,700 β (beta) were observed in equimolar amounts. They are the acidic and basic polypeptides of oat globulin consisting of six subunits (6 α and 6 β). Several studies have found similar subunit sizes for globulin later using sedimentation equilibrium centrifugation and other chromatographic methods (Brinegar and Peterson 1982).

3.5.2. Electrophoresis

Electrophoresis is a major method that several chemists have utilized for the characterization of grain storage proteins. Whatever extraction methods are used, subsequent electrophoretic analyses are inevitable for identifying different classes of protein and their molecular weight pattern. These analyses not only help identify protein fractions at individual level, but also help elucidate the complexity and overlap between several protein fractions, their size, and solubility. Several electrophoretic methods for separating and characterizing oat storage proteins are described in detail in this section.

3.5.2.1. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). SDS-PAGE is a very common method that is used routinely for oat storage protein separation and characterization. It is also called the Laemmli method after Laemmli, who introduced SDS-PAGE in scientific studies for the first time (Laemmli 1970). This method was used to characterize the relative molecular mass, the relative abundance of oat globulin proteins in the grain, and the distribution of different solubility fractions such as albumin and avenin proteins. Table 1 shows the major developments in oat protein characterization using the SDS-PAGE technique.

Starch gel electrophoresis and moving boundary electrophoresis were the two other electrophoretic methods that were initially used for oat protein separation. Both methods were mainly used for the cultivar identification of oat using isozymes. Using starch gel electrophoresis, α, β, and γ groups of avenin protein were identified (Kim, Charbonnier, and Mosse 1978, Wu et al. 1972, Draper 1973, Waldschmidt-Leitz and Zwisler 1963). In addition, a significant variability of avenin pattern between species under the same cultivar was also identified. The molecular size and charge heterogeneity of avenin differs with ploidy increases, which suggests polymorphism of avenin that was also revealed by electrophoresis (Kim, Charbonnier, and Mosse 1978, Kim, Saur, and Mosse 1979). For the characterization of admixture detection in meal samples, SDS-PAGE of avenin provides very high reliability compared with albumins and globulins protein fractions of oat (Dvořáček, Čurn, and Moudrý 2003). Acid-PAGE is another electrophoretic method that is mainly used for oat cultivar identification using high-molecular-weight protein components (Brinegar and Peterson 1982, Adeli and Altosaar 1983).

Using two-dimensional electrophoresis, the differences in electrophoretic mobility of globulin under reducing and non-reducing condition was identified. In reducing conditions, two bands were clearly visible with the molecular weight of 19–25 kDa and 35–42 kDa, which corresponds to α and β subunits of globulin (BURGESS and MIFLIN 1985, Peterson 1978). When the separation was carried out in the non-reducing condition, a band with a molecular weight above 55kDa was noticed. The molecular weight of unreduced α and β polypeptides was approximately 53–58 kDa was also confirmed by 2D-PAGE (Matlashewski et al. 1982).

3.5.2.2. Capillary zone electrophoresis (CZE). Separation of cereal proteins, especially oat prolamin and glutelins, was characterized with capillary zone electrophoresis (CZE) and capillary electrophoresis (CE). Using CZE, Lookhart et al. (1999) separated oat proteins and confirmed that 80 percent of oat proteins are globulins (Bean and Lookhart 1999, Lookhart and Bean 1996). Although it is a complementary method for Acid-PAGE (A-PAGE), CZE separates oat proteins with high resolution within 2–5 minutes, which is faster than A-PAGE. In addition, the CZE can separate albumins and globulins from the remaining avenin fraction of oat. Thus, it offers us one of the best cultivar identification methods, which is using avenin protein fraction (Lookhart and Pomeranz 1985). Using aspartic acid or low conductive buffers in CZE with phosphate-glycine gives improved cereal storage protein analysis (Bean and Lookhart 1999, Capelli et al. 1998). In addition, using isoelectric iminodiacetic acid (IDA) and acetonitrile (ACN) in CZE gave high resolution of proteins resolved in less than 3 minutes. This is faster than any other existing electrophoretic methods and helped to avoid urea (make contamination and take much more time to clean the apparatus) in the extraction buffer (Bossi and Righetti 1997, Righetti et al. 1999).

3.5.2.3. Isoelectric focusing (IEF). Isoelectric focusing was mostly used for the characterization of avenin and glutelins. Three main groups of avenins, α, β and γ, were observed at both acidic and basic pH using the IEF method (Ma and Harwalkar 1984). Horizontal and vertical IES characterized carboxymethylated globulin, carboxyamidomethylated globulin, and carboxymethylated α and β polypeptides (Lookhart and Bean 1995).

Using zonal isoelectric precipitation and focusing, the hexameric nature oat globulin subunits were characterized (Burgess et al. 1983). In IEF, the large and small subunits of oat globulin showed similarities between the globulin proteins of legume and certain dicotyledonous plants including the family of Crucifereae and Cucurbitaceae (Derbyshire and Boulter 1976).

3.5.3. Chromatography

Chromatography has been used for the isolation and characterization of cereal proteins since the 1960s (Hubner and Wall, 1966), and chromatographic methods that provide superior separations were developed for the distinct characterization of oat proteins or polypeptides in the early 1980s. Many methods based on the chromatographic technique have been used to quantify and fractionate of oat protein, as displayed in Table 2. The charge properties, size and PI differences of different protein fractions have been successfully utilized for the separation using chromatographic methods. Using column chromatography, solubility fractions such as albumins (soluble in water), globulins (soluble in saline solutions, but insoluble in water), prolamins (soluble in aqueous solutions of alcohol), and

Table 1. Major developments occurred in oat protein fraction identification using SDS-PAGE.

Methods	Extraction medium	Fractions identified	% of Acrylamide	References
SDS-PAGE	1 mol/L NaCl, 0.05 mol/L Tris buffer at pH 8.5	Globulin	10%	(Luthe and Peterson 1977)
SDS-PAGE	1 mol/L NaCl, 0.05 mol/L Tris at pH 8.5	Globulin	4–10%	(Peterson 1978)
SDS PAGE	1 mol/L NaCl and 0.1 **N** acetic acid, 70% ethanol	Albumin, globulin, and avenin	5%	(Wu et al. 1977)
SDS-PAGE & Starch gel electrophoresis.	Ethanol, H_2O mixture	Prolamin α and β globulin	—	(Kim, Charbonnier, and Mosse 1978, Kim, Saur, and Mosse 1979)
SDS-PAGE	0.1 mol/L sodium acetate, 0.1N acetic acid and sucrose.	Esterase, peroxidase enzymes	7.5%	(McDonald 1980)
SDS- PAGE	70% ethanol	Avenin	7%	(Laurière and Mossé 1982)
SDS-PAGE, 2D-electrophoresis	6 mol/L urea & Tris buffer	Globulin	6–12%	(Brinegar and Peterson 1982)
SDS-PAGE	1 mol/L NaCl, 0.05 mol/L Tris at pH 8.5	Globulin	15–18%	(Matlashewski et al. 1982)
SDS-PAGE, Ethanol	0.5 mol/L NaCl, 45% ethanol	Globulin, albumin, and prolamin	6%	(Pernollet, Kim, and Mosse 1982)
SDS-PAGE	0.05 mol/L Tris/Hcl containing 1.0 mol/L NaCl at pH 8.5	Globulin, albumin, and prolamin	12–14%	(Burgess et al. 1983)
SDS-PAGE	1 mol/L NaCl and Tris buffer	Invitro translated protein	12–14%	(Adeli and Altosaar 1983)
SDS-PAGE	Sucrose gradient centrifugation	Protein bodies separated from aleurone layers	12%	(Donhowe and Peterson 1983)
SDS- PAGE	NaCl, 10 mmol/L 2-mercaptoethanol, 0.2 mmol/L m phenylmethylsulfonyl fluoride in 0.1 mol/L Tris, pH 8.5	Globulin	12–15%	(Walburg and Larkins 1983)
IEF & SDS-PAGE	1 mol/L NaCl and Tris buffer	Prolamin & Globulin	14%	(Robert et al. 1983)
SDS-PAGE	1 mol/L NaCl and Tris buffer	Globulin, albumin, and prolamin	12–14%	(Robert, Nozzolillo, and Altosaar 1985)
SDS-PAGE	70% ethanol	Avenin	7.5%	(Lookhart and Pomeranz 1985)
SDS-PAGE	50 mmol/L Tris-Hcl 1 mol/L NaCl, and 1- Mercaptoethanol and 0.1 mol/L NaOH	Globulin	10%	(Luthe 1987)
SDS-PAGE	1.0 mol/L NaCl	Globulin	7.5%	(Ma and Khanzada 1987b, Ma et al. 2000)
SL-PAGE	70% ethanol	Avenin N9 and Avenin 3	20%	(Egorov 1988)
Acid –PAGE & SDS-PAGE	Ethanol-H_2O	Avenin N9 and Avenin 3	12.6%	(Pernollet, Kim, and Mosse 1982)
SDS-PAGE	1 mol/L NaCl, 0.05 mol/L Tris	Oat bran globulin	12%	(Nnanna and Gupta 1996)
Acid -PAGE	70% ethanol	Prolamin	13–17%	(Portyanko, Sharopova, and Sozinov 1998)
SDS-PAGE	50% ethanol	Prolamin	10%	(Tatham et al. 2000)
SDS-PAGE	Na-Phosphate buffer, 25% 2- Chloro-ethanol	Globulin, albumin, and avenin	—	(Dvořáček, Čurn, and Moudrý 2003)
SDS-PAGE	0.2 mol/L Na phosphate buffer	Globulin, albumin	8–25%	(Runyon et al. 2013, Runyon et al. 2015)

glutelins (soluble in dilute acid or alkali) (Osborne 1907) were resolved and each were found to have unique polypeptide composition. Anion and cation exchange chromatography, gel filtration chromatography, exclusion chromatography, and high-pressure liquid chromatography are the commonly used chromatographic methods for separation of oat proteins.

3.5.3.1. Anion and cation exchange chromatography. The size and charge difference between avenin and globulin fractions were studied mainly using ion exchange chromatography with different columns, where the ionizable side chain is utilized for separation. Brinegar and Peterson (1982) separated α (alpha) and β (beta) subunits of globulin fraction using anion exchange chromatography with diethyl amionoethyl (DEAE) Sepharose CL-6B column in 6M urea (Brinegar and Peterson 1982). Walburg and Larkins (1983) obtained similar separations using DEAE-Sephadex along with 1 and 2D SDS-PAGE (Walburg and Larkins 1983). The results obtained from ion exchange chromatography, SDS-PAGE revealed the dimeric form of α, and β globulin associated with a disulphide bond. In addition, α and β polypeptides from reduced and carboxymethylated oat globulin were characterized using ion exchange chromatography (Brinegar and Peterson 1982, Pernollet, Kim, and Mosse 1982, Kim, Charbonnier, and Mosse 1978).

Avenin fractions were separated with strong cation exchange column and the isolated protein bodies in an electrophoretic gel revealed its location within the protein bodies. Several avenin protein fractions, such as Avn-2, Avn-5, Avn-6, and Avn-9, were characterized using a combination of cation exchange and RP-HPLC. In agreement with the behavior in ion exchange chromatography, these identified avenin fractions were quite different in net charge at low pH, with the exception of Avn-7 and Avn-8 (Wieser and Belitz 1989, Egorov, Musolyamov, Kochergin, et al. 1994, Pernollet et al. 1989)

3.5.3.2. Gel filtration chromatography. Gel filtration chromatography (also called gel permeation, molecular exclusion, or size-exclusion chromatography) separates proteins primarily by size (Bietz 1979). It is known to be useful for the comparison of different varieties (elution profile) or the effect of agronomic or other factors on solubility fraction of oat proteins. Unmodified oat proteins, as well as acetylated and succinylated oat

Table 2. Major developments occurred in oat protein fraction identification using various chromatographic methods.

Chromatographic methods	Extraction solvent	Monitored fraction	References
Ion exchange	Ethanol/H_2O mixture	Avenin	(Kim, Charbonnier, and Mosse 1978)
Ion exchange	Urea & Tris buffer	α and β globulin	(Brinegar and Peterson 1982)
Column	0.2M NaCl	Globulin	(Burgess et al. 1983)
HPLC & PAGE	–	Avenin	(Lookhart 1985)
RP-HPLC & PAGE	70% Ethanol	Avenin	(Lookhart and Pomeranz 1985)
Ion exchange, RP-HPLC (2D-HPLC)	Ethanol/H_2O mixture	Avenin	(Pernollet et al. 1989)
RP-HPLC	–	Avenin	(Wieser and Belitz 1989)
HPLC-PAGE	1.0 mol/L NaCl	Globulin	(Harwalkar and Ma 1987)
HPLC	2M NaCl	Globulin	(Robert et al. 1983, Burgess et al. 1983)
RP-HPLC,IE-HPLC	70% ethanol	Avenin	(Egorov 1988)
RP-PHLC		Avenin	(Lapvetelainen, Bietz, and Huebner 1995)

proteins, were also analyzed using this method (Ma and Wood 1987a). A combination of gel filtration chromatography and SDS-PAGE has been used to analyze the dissociation of aggregated and oligomeric oat proteins by acid hydrolysis and molecular weight determination of oat bran globulin from oat protein isolates (Nnanna and Gupta 1996). Because of long retention time and poor resolution (if the protein fraction is associated with many individual components) are the disadvantages of using gel filtration.

3.5.3.3. Exclusion chromatography. Exclusion chromatography is known to be a useful technique especially in the purification and preliminary fractionation of crude extracts of proteins. Using gel filtration media (Sephadex G-100 and SP- Sephadex c-50), electrophoretic constituents of alcohol-soluble protein of oats (Avenin) with the same molecular weight pattern were identified. In addition, end terminal amino acid threonine and the ancestral relationship of oat avenin with wheat and barley were identified when exclusion gel chromatography was combined with SDS-PAGE. Also, the amino acid composition of avenin proteins extracted from different cultivars were analyzed and the species relationship between cultivars were identified (Kim, Charbonnier, and Mosse 1978). However, this method less common nowadays because of the poor reproducibility in molecular weight calculations. Another disadvantage of using exclusion chromatography is that it has a lower peak capacity due to the inability of chromatographic column to resolve compounds with molecular sizes above the separation limit.

3.5.3.4. High-performance liquid chromatography (HPLC). The use of HPLC in oat protein characterization started in the late 1980s and has mainly been used for avenin characterization. Prior to the 1980s, SDS-PAGE of avenin was used for oat cultivar identification because of a distinct banding pattern of avenin in electrophoretic gel for different ploidy (diploid, tetraploid and hexaploid) level. However, if the coefficient of parentage between two cultivars is higher, it always produces a similar avenin pattern in the gel. It is difficult to differentiate between them using the SDS-PAGE pattern alone. Since 1980s, chromatographic methods providing superior separations have been developed. These methods have been termed HPLC (High-Performance Liquid Chromatography) and have been successfully used for oat protein separation, detection of quality differences between fractions, and variety identification. 1D- and 2D-HPLC (ion exchange followed by reverse-phase (RP) HPLC) was also used for identifying peptides from protein mixtures of oat (Pernollet et al. 1989). A combination of HPLC and SDS-PAGE or 2D-HPLC was successfully used for characterizing intra- and inter-genic specific heterogeneity and determining the degree of homology for detaining species relationship and cultivar identification in oats (Pernollet, Kim, and Mosse 1982, Wieser and Belitz 1989, Egorov, Musolyamov, Andersen, et al. 1994). Oat globulins and glutelins have also been characterized with HPLC (Lapvetelainen, Bietz, and Huebner 1995).

HPLC is one the main analytical methods for analyzing cereal protein and has been found to have numerous advantages. One of the main advantages is its resolution compared with conventional chromatography and electrophoresis. Excellent reproducibility and sample recovery can be obtained due to controlled and accurate flow rate in the system. It requires less "art" compared with electrophoresis and most HPLCs are easy to operate and can be learned in a few hours. The complexity and high cost of HPLC machinery are the two mains limitation of using HPLCs. However, it remains a widely used method all over the world in protein characterization.

3.6. Detection methods

3.6.1. Immunological methods

A number of immunoassay methods have been devised for the analysis of oat protein fractions, their synthesis, organelle-based aggregation, and quantification. Commonly used direct assays are radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and immune staining methods.

The radio immunological assay (RIA) is an *in vitro* assay technique used to measure very small amounts of substances that are in the micromolar to nanomolar range in a sample (Hawker 1973). RIA makes it possible to quantify oat globulin content in developing seeds during different days post-anthesis (Luthe and Peterson 1977, Colyer and Luthe 1984). They also used RIA to study *in vitro* synthesis of globulins and albumins using isolated proteins from endoplasmic reticulum (ER) with discontinuous sucrose gradient. Using this method to calculate the relative proportion of globulin to total seed storage protein and found that it accounted for 75 percent with a molecular weight range of 60,000–68,000 Daltons (Walburg and Larkins 1983).

Table 3. Details of analytical methods applied to the analysis of oat proteins.

Title of the article	Place published	Major Methods/ technique used	Significant outcome/achievements/ explained or revealed	References
Amino acid composition of oat groats	U.S	Beckman 121 automatic analyzer	The most comprehensive report of the amino acid composition of oat groats (289 samples)	(Pomeranz, Robbins, and Briggle 1971)
Oats and Their Dry-Milled Fractions: Protein Isolation and Properties of four Varieties	U.S	Beckman SpinCo model 120 amino acid analyzer.	Nutritive value of dry milled fractions	(Wu et al. 1972)
Oat Protein Concentrates from a Wet-Milling Process: Preparation.	U.S	Micro-Kjeldahl	Explained a simple process for preparing oat protein concentrate	(Cluskey et al. 1973)
Amino acid profiles of chemical and anatomical fractions of oat grains	UK	Electrophoresis, amino acid analyzer	Explained the amino acid composition of oat globulin	(Draper 1973)
Protein content and amino acid composition of oat species and tissues	US	Kjeldahl, Beckman 121 automatic analyzer	Interspecies differences between amino acid is greater than differences in oat groat compositions	(Pomeranz, Youngs, and Robbins 1973)
Protein electrophoresis as an aid to oat variety identification	US	Slab gel electrophoresis.	Oat varieties identified based on isozyme pattern	(Singh, Jain, and Qualset 1973)
Oat Protein Concentrates from a Wet-Milling Process: Composition and Properties.	US	Beckman SpinCo model 120 amino acid analyzer	Explained good nitrogen solubility and hydration capacity of oat protein concentrates	(Wu et al. 1973)
Protein concentrates from oat flours by air classification of normal and high-protein varieties	US	Beckman SpinCo model 120 amino acid analyzer, Micro-Kjeldahl, Air classification by Pillsbury Laboratory model classification	Explained Air classification method provide protein concentrates with good amino acid composition	(Wu 1973)
Correlations among chemical and agronomic characteristics in certain oat cultivars and selections	US	Kjeldahl	Correlation between protein percent, lipid and kernel weight were found.	(Forsberg, Youngs, and Shands 1974)
An evaluation of the nutritive value of new high protein oat varieties (cultivars)	US	Macro-Kjeldahl	Explained the protein quality of oat protein compared with egg white	(Maruyama et al. 1975)
Changes in Nitrogen and Carbohydrate Fractions in Developing Oat Groats	US		Calculated the Proportions of albumin, globulin, avenin and glutelins and their changes in groat maturation	(Peterson and Smith 1976)
Cell-free Synthesis of Globulin by Developing Oat (Avena sativa L.) Seeds	US	Cell free protein synthesis system and analysis, SDS-PAGE	Explained the membrane bound as well as free polysomal synthesis of oat globulin	(Luthe and Peterson 1977)
Protein isolate from high protein oats- preparation, composition and properties.	US	Alkaline extraction of protein & amino acid analysis by Beckman Spinco Model 121 amino acid analyser.	Increased hydration capacity, good emulsifying capacity and good emulsion stability of protein isolates were noticed.	(Wu et al. 1977)
Heterogeneity of Avenin, the oat protein fractionation, mol.wt and amino acid composition	France	Ion exchange chromatography(SP/ Sephadex C50) Exclusion chromatography G/100 Chromatography on SP Sephadex, starch gel electrophoresis	Two novel fractions of oat avenin and a common ancestral gene for avenin between cereals were identified.	(Kim, Charbonnier, and Mosse 1978)
Subunit structure and composition of oat seed globulin	US	Ultracentrifugation, SDS-PAGE, amino acid analysis	Hexameric structure of oat globulins has been proposed	(Peterson 1978)
Some features of the inheritance of avenins, the alcohol soluble proteins of oat	US	Gel electrophoresis	Interspecific variability of prolamin protein fractions were identified and suitability of using avenin for phylogenetic studies was confirmed	(Kim, Saur, and Mosse 1979)
Oat cultivar characterization using electrophoresis	US	SDS-PAGE, Spectrophotometer.	Rapid identification of oat cultivar based on esterase and peroxidase enzymes was confirmed.	(McDonald 1980)
Separation and characterization of oat globulin polypeptide	US	Isoelectric focusing Sedimentation equilibrium centrifugation, Anion exchange chromatography, isoelectric focusing	Heterogeneity in α and β polypeptide of oat globulin was identified	(Brinegar and Peterson 1982)
Polyacrylamide gel-urea electrophoresis of cereal prolamins at acidic pH	France	Polyacrylamide gel-urea electrophoresis	Prolamin fractions of different cereals has been characterized including oat avenin	(Laurière and Mossé 1982)
Invitro synthesis of oat globulin	UK	Invitro synthesis of oat globulin using wheat germ cells, scintillation spectrometry, SDS-PAGE	The 12S oat globulin resembles the legume 11 S proteins in that the two types of subunits are associated into dimers stabilized by disulfide bonds.	(Matlashewski et al. 1982)

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Table 3. (*Continued*)

Title of the article	Place published	Major Methods/ technique used	Significant outcome/achievements/ explained or revealed	References
Characterization of storage proteins extracted from Avena sativa seed protein bodies	France & South Korea	Differential Centrifugation, optical microscope equipped with phase contrast and polarized light measurements.	Isolation of protein bodies, albumin, globulins and acetic acid soluble glutelins were carried out	(Pernollet, Kim, and Mosse 1982)
Role of Endoplasmic Reticulum in Biosynthesis of Oat Globulin Precursors	Canada	Immunoprecipitation	Explained the synthesis of oat storage proteins in RER and later proceed in to protein bodies	(Adeli and Altosaar 1983)
Characterization of oat seed globulin	UK	ultracentrifugation, SDS-PAGE, gel filtration, zonal isoelectric precipitation, sucrose gradient ultracentrifugation, using marker proteins,	Explained the synthesis of 3 major components of oat globulins (3s, 7s and 12s) and similarities of oat globulins with seeds of legumes and other dicotyledonous plants were noticed.	(Burgess et al. 1983)
Isolation and characterization of oat aleurone and starchy endosperm protein bodies	US	Electron microscopy, SDS-PAGE, Sucrose density gradient centrifugation.	Both aleurone and starchy endosperm protein bodies contain oat globulin and prolamin. Protein bodies and goblin have shown similar amino acid composition	(Donhowe and Peterson 1983)
Total Solubilization of Groat Proteins in High Protein Oat (Avena sativa L. cv. Hinoat): Evidence that Glutelins are a Minor Component	US	Sequential extraction of oat globulin, SDS-PAGE	Globulin is the predominant protein fraction and no major polypeptide associated with glutelins was identified	(Robert et al. 1983)
Isolation and characterization of oat globulin messenger RNA	US	Invitro translation of poly (A)-RNA, immuno precipitation of invitro labelled oat globulin	Explained a putative precursor of oat globulin (18 S), is may be a template for 58 to 60 KDa of oat globulin.	(Rossi and Luthe 1983)
Oat seed globulin- subunit characterization and demonstration of its synthesis as a precursor	US	SDS-PAGE, 2D electrophoresis	The globulin subunits are synthesized on rough endoplasmic reticulum membranes and are proteolytically processed in to acidic and basic polypeptides before deposition in to the vacuole	(Walburg and Larkins 1983)
The composition of oat husk and its variation due to genetic and other factors	Ireland	AOAC methods	Oat husk contain only 3–4% of total protein	(Welch, Hayward, and Jones 1983)
Functional properties of acylated oat protein	UK	Invitro digestibility, gel filtration chromatography and amino acid analysis,	Acetylation increased the functional properties of oat proteins	(Ma 1984)
Chemical characterization and functionality assessment of oat protein fractions	UK	Column chromatography, isoelectric focusing, differential scanning colorimetry,	Solubility fractions of oat groats were identified	(Ma and Harwalkar 1984)
The Localization of Oat (Avena sativa L.) Seed Globulins in Protein Bodies	UK	SDS-PAGE, immuno diffusion, sucrose gradient ultra-centrifugation	Localization of globulin and avenin are occurs at different protein bodies	(BURGESS and MIFLIN 1985)
Characterization of developing oat seed mRNA evidence for many globulin mRNAs	UK	SDS,IEF-SDS gel electrophoresis, immunoprecipitation	A 60 KDa precursor globulin and heterogeneity is polypeptide chains were identified	(Fabijanski, Matlashewski, and Altosaar 1985)
Identification of oat cultivars by combining polyacrylamide gel electrophoresis and reversed phase high performance liquid chromatography	US	PAGE-RP HPLC	Combined use of PAGE and RP-HPLC identified as a powerful tool for cultivar identification	(Lookhart 1985)
Characterization of oat species by polyacrylamide gel electrophoresis and high performance liquid chromatography of their prolamin proteins	US	PAGE and HPLC	Heterogeneity in Avenin proteins between cultivars were identified	(Lookhart and Pomeranz 1985)
Homology between rice glutelins and 12 S globulin	UK	Western blot	Percent of homology of oat globulin with rice glutelins is more than compared with legume globulins	(Robert, Nozzolillo, and Altosaar 1985)
The Variation in Chemical Composition of Swedish Oats	Sweden	AOAC methods	The variations in protein, lipid, dietary fiber, starch, ash and carbohydrate contents were determined	(Åman 1987)

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Table 3. (*Continued*)

Title of the article	Place published	Major Methods/ technique used	Significant outcome/achievements/ explained or revealed	References
Study of Thermal Properties of Oat Globulin by Differential Scanning Calorimetry	Canada	Differential scanning colorimetry	Importance of hydrophobic interaction in the stability of oat globulin has identified.	(Harwalkar and Ma 1987)
Storage protein synthesis during oat (*Avena sativa L.*) seed development	US	Immunoprecipitation, in vivo labelling, SDS-PAGE	Oat globulin mRNA was the most abundant in 15 th day of anthesis, which is the peak time of storage protein synthesis.	(Luthe 1987)
Functional properties of deamidated oat protein isolates	UK	Gel filtration, Chromatography	Extensive dissociation without the cleavage of peptide linkage and increase in functional properties after deamidations were observed	(Ma and Khanzada 1987a)
Functional properties of oat proteins modified by acylation, trypsin hydrolysis or linoleate treatment	UK	Gel chromatography, amino acid analysis	Dissociation of oat polypeptides by succinylation and improved functional properties were identified	(Ma and Wood 1987b)
The amino acid sequence of the "fast" avenin component (Avena sativa L)	Moscow	Ion exchange high performance liquid chromatography, Amino acid sequence of avenin	The first complete protein sequence of the one of the avenin components (designated N9) which migrates faster than the others when examined in SDS-PAGE and which is present in the majority of oat varieties.	(Egorov 1988)
Study of thermal denaturation of oat globulin by ultraviolet and fluorescence spectrophotometry	UK	UV spectroscopy, fluorescence emission spectrometry, amino acid analysis	Heat treated globulin have significantly higher surface hydrophobicity than the non-heat treated globulin	(Ma and Harwalkar 1988)
Thermal gelation of oat globulin	Canada	Differential scanning Colorimetry	Explained the role of fatty acids and chemical forces involved in gelling properties of globulin	(Ma, Khanzada, and Harwalkar 1988)
Some functional properties of acetylated and succinylated oat protein concentrates and a blend of succinylated oat protein and whey protein concentrates	Canada	Size exclusion-HPLC	Improved nitrogen solubility, emulsifying properties, water and fat binding capacity were identified in acetylated oat proteins	(Ponnampalam et al. 1988)
Analysis of avenin proteins and the expression of their mRNAs in developing oat seeds	US	Construction of cDNA library, hubrid arrest translation of avenin mRNAs, sequence analysis of avenin clones, hybridization analysis of oat genomic DNA.	Increased availability of avenin mRNA than globulin mRNA during oat seed development was explained. Two repetitive regions in avenins mRNA was also observed	(Chesnut et al. 1989)
Immunolocalization of avenin and globulin storage proteins in developing endosperm of *Avena sativa L*	USA	Gel electrophoresis, Western blotting, SDS-PAGE, double labeled immuno localization, anti-globulin immuno staining	Endoplasmic reticular aggregation of prolamins and vacuolar aggregation of globulins were identified	(Lending et al. 1989)
2D-HPLC separation, electrophoretic characterization and N-terminal sequences of oat seed prolamins	France	2D-HPLC, SDS-PAGE, N- terminal sequencing	Microheterogenity of true avenin was identified. .A pseudo helical structure of avenin was proposed.	(Pernollet et al. 1989)
Amino acid composition of avenins separated by reversed- phase high performance liquid chromatography	Germany	RP-HPLC	Explained the thirty components of oat avenin components and 3 subgroups of avenin	(Wieser and Belitz 1989)
Physicochemical properties of alkali-treated oat globulin	UK	Differential scanning colorimetry, SDS-PAGE, HPLC, proteolysis	Explained the partial denaturation and alterations in the oligomeric structure (degradation and aggregation) of globulin protein by alkali treatments	(Ma, Harwalkar, and Paquet 1990)
Isozyme Variation in Cultivated Oat and Its Progenitor Species *Avena sterilis L*	US		Explained the use of isozyme for effective identification of oat cultivars	(Murphy and Phillips 1993)
The complete amino acid sequence and disulphide bond arrangement of oat alcohol-soluble avenin-3	Russia & Danmark	CNBr and trypsin generated peptide sequencing, HPLC	Explained the degree of similarity between prolamins is due to the cysteine residue regions with disulfide bonds	(Egorov, Musolyamov, Andersen, et al. 1994)
Isolation, characterization by mass spectrometry and partial amino acid sequencing of avenins	Denmark	IE-HPLC followed by RP-HPLC	11 avenin (10 major and 1 minor) components -was detected by acid- PAGE after extraction with aqueous ethanol. Explained the low heterogeneity of avenin compared to wheat and barley	(Egorov, Musolyamov, Kochergin, et al. 1994)

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Table 3. (*Continued*)

Title of the article	Place published	Major Methods/ technique used	Significant outcome/achievements/ explained or revealed	References
Reversed-Phase High-Performance Liquid Chromatography of Oat Proteins: Application to Cultivar Comparison and Analysis of the Effect of Wet Processing	Finland	RP-HPLC	Explained the potential of RP-HPLC technique in oat cultivar identification	(Lapvetelainen, Bietz, and Huebner 1995)
Rapid differentiation of oat Cultivars and Rice Cultivars by Capillary Zone Electrophoresis	US	Capillary Zone Electrophoresis.	The first report for rice and oats have been separated by CZE.	(Lookhart and Bean 1995)
High performance capillary electrophoresis of cereal proteins	USA	High performance capillary electrophoresis.		(Bean, Bietz, and Lookhart 1998)
Characterization of European oat germ plasm: allelic variation at complex avenin loci detected by acid polyacrylamide gel electrophoresis	USA	SDS_PAGE	Explained the allelic variation of avenin in European oat cultivars	(Portyanko, Sharopova, and Sozinov 1998)
Ultrafast capillary electrophoretic analysis of cereal storage proteins and its applications to protein characterization and cultivar differentiation	US	Ultrafast capillary electrophoresis	Cereal storage protein from several types of cereals could be analyzed with in short time (2-8 min) with extremely high resolution and repeatability	(Bean and Lookhart 2000)
Genotypic and environmental effects on oat milling characteristics and groat hardness	US			(Doehlert and McMullen 2000)
Raman spectroscopic study of oat globulin confirmation	China	FT/ Raman Spectroscopy	Revealed the effect of pH, chemotropic salts, protein perturbants and heating on the primary and secondary structure oat globulin confirmation	(Ma et al. 2000)
Study of oat globulin conformation by Fourier transform infrared spectroscopy	China	FTIR	Explained the formation of beta turn, beta sheets, alpha helices, random coils and protein unfolding of globulin	(Ma, Rout, and Mock 2001)
Suitability of oat seed storage proteins markers for identification of cultivars in grain and mixed flour samples	Czech republic	Dual slab electrophoresis SDS-PAGE	Avenins characterized under SDS-PAGE conditions, are reliable implements for the identification of oat cultivars	(Dvořáček, Čurn, and Moudrý 2003)
Genetic variability for chemical components in sixty European Oat cultiars	Italy	Microkjeldahl, gravimetric method for TDF	A large variability among Italian genotypes and European germplasm of oat cultivar was identified.	(Redaelli, Sgrulletta, and Stefanis 2003)
Effect of variety, nitrogen fertilizer and various agronomic factors on the nutritive value of husked and naked oats grain	UK	Amino acid analysis, dietary fiber analysis	Explained the differences in nutritional profile of Naked and Husked oat cultivars	(Givens, Davies, and Laverick 2004)
Study of Thermal Aggregation of Oat Globulin by Laser Light Scattering	China	Size exclusion chromatography combined with one line multiangle laser light scattering (MALLS) and quasi-elastic light scattering (QELS)	Aggregation of oat globulin, dissociation of hexameric form, formation of soluble and insoluble aggregates of globulin upon heating were explained	(Zhao, Mine, and Ma 2004)
Relationships among agronomic traits and grain composition in oat genotypes grown in different environments	US	HPLC, Calcoflour, infrared transmittance	Explained the variances associated with genotype for yield, alpha-tocopherol, and the avenanthramides.	(Peterson et al. 2005)
Influence of physical grain characteristics on optimal rotor speed during impact dehulling of oats	US	Impact dehuller, Mass digital image analysis, Bulk density analysis	Influence of physical grain characteristics on optimal rotor speed during impact dehulling of oat was analysed	(Doehlert and Wiessenborn 2007)
Analysis of free amino acids in cereal proteins	Sweden	Compared the results of Phenomenex and Biochrom EZ-Faast method of amino acid analysis.	Rapid estimation of amino acid using Phenomenex and Biochrom EZ-Faast techniques	(Mustafa et al. 2007)
Composition, secondary structure and self-assembly of oat protein isolate	China	Fourier transform infrared spectroscopy (FTIR), and tapping mode atomic force microscopy (TP-AFM).	Showed the amino acid compositions, secondary structure, and self-assembly of oat protein isolate (OPI) purified from a high-protein Chinese oat	(Liu et al. 2009)
Effects of succinylation and deamidations on functional properties of oat protein isolate	Iran	AOAC methods, SDS-PAGE	Foaming capacity of oat protein isolate increased after deamidations, whereas succinylation decreased it. Water- and oil-binding capacity, in both modified oat proteins, was higher than those of the native oat protein isolate.	(Mirmoghtadaie, Kadivar, and Shahedi 2009)

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Table 3. (*Continued*)

Title of the article	Place published	Major Methods/ technique used	Significant outcome/achievements/ explained or revealed	References
Groat proportion in oats as measured by different methods: Analysis of oats resistant to dehulling and sources of error in mechanical dehulling	USA		A groat proportion value calculated from mean kernel mass after different dehulling process did not showed any significant differences from the hand dehulling value	(Doehlert, McMullen, and Riveland 2010)
Discrimination of oats (Avena sativa L.) cultivars using isozyme markers	India	SDS-PAGE	Variation of band intensity in isozyme peroxidase polyphenol oxidase, esterase and amylase can be used for oat cultivar identification	(Balamurugan 2014)
Characterization of oat proteins and aggregates using asymmetric flow field-flow fractionation	Sweden	Asymmetrical flow filed flow fractionation, elemental analyser, SDS-PAGE	Monomeric and aggregate oat proteins were characterized	(Runyon et al. 2013)
The effect of heat treatment on the soluble protein content of oats	Sweden	Asymmetrical flow filed flow fractionation, elemental analyser, SDS-PAGE, amino acid analysis	Albumin and water soluble prolamin heat sensitive and globulin is thermally stable	(Runyon et al. 2015)

Enzyme-linked immunosorbent assay (ELISA), or Enzyme immuno assay (EIA), is a plate-based method designed for detecting protein, peptides, and hormones. In oats, it is mainly used for characterizing globulin and avenin protein synthesis, their translocation difference in different organelles, posttranslational modifications, and the influences of pH and ionic strength on synthesis and translocation. By using ELISA, the globulin/avenin ratio of 26.1 (by weight) or 10.1 (molar basis) in mature seeds were quantified (Chesnut et al. 1989, Capouchová, Petr, and Krejcírová 2006). Quantification, mRNA translation and immunoprecipitation of translated products oat globulins have also been studied (Rossi and Luthe 1983).

Immunostaining is an antibody-based method for detecting specific protein in a sample. Using the immunolocalization of avenin and globulin storage proteins in developing endosperm with double-labeled immune-gold staining of oat endosperm has been studied. This study revealed the direct aggregation and deposition of globulin in vacuole at higher percentage than in the RER (rough endoplasmic reticulum). By contrast, avenin aggregates more in RER than with vacuolar aggregation (Lending et al. 1989). The advantages of immunoassay methods are that small number of seeds are needed and that problems with cross-contamination from other protein fraction are eliminated. However, denaturation and deactivation of protein by free radicals and nonspecific binding of closely related proteins are the limitations of frequently using immunoassay methods (Saha, Bender, and Gizeli 2003).

3.6.2. Use of enzymes

Isozymes are one of the most widely used biochemical markers in plant breeding and genetics. Singh et al. (1972) successfully used intra- and inter-varietal isozyme variation for varietal identification in oats. By using the position, type, and the staining intensity of the isozyme bands of Esterase (E), Leucine amino peptidase (LAP), anodal and cathodal peroxidase (APX, CPX), 10 oat cultivars were differentiated (Murphy and Phillips 1993, Balamurugan 2014). Conditions for successful use of isozyme in varietal identifications are distinct staining characteristics and a cultivar-specific banding pattern for each of the enzymes under study. The enzyme systems are usually examined under horizontal starch gel electrophoresis; the entire analysis only takes one day to complete and does not require any elaborate instruments, making it an easy and relatively stable method of cultivar identification (Singh, Jain, and Qualset 1973). However, if the cultivars are generated from a single variety that used to be homogenous and does not warrant any differences between enzymes in a gel, more sophisticated studies are required.

3.6.3. Differential scanning colorimetry (DSC)

DSC is a thermoanalytical technique for studying the stability of proteins in their native form and observing what changes happen to the proteins when they are heated (Gill, Moghadam, and Ranjbar 2010). Changes in peak width in DSC measurements due to an increase or decrease in temperature are sufficient for understanding whether the preheated protein has assumed a structure with higher thermal stability or not. It is quite suitable for the characterization of plant proteins that are not readily soluble in aqueous buffers. Using DSC, the thermal behavior of oat globulin has been characterized and the differences in native and heat treated oat globulin has been analyzed (Ma, Khanzada, and Harwalkar 1988). Changes in enthalpy with heating time, degree of denaturation and formation of soluble and insoluble aggregates oat protein fractions have also been studied (Ma and Harwalkar 1988).

3.6.4. Microscopy

A variety of microscopic techniques are available for studying the microstructure of cereals. Microscopy provides high resolution, chemical specificity, and sensitivity compared to other detection methods (Autio & Salmen Kallio-Martila, 2001). These methods were mainly used to locate the organelles of protein synthesis in oats. Phase contrast microscopy has shown that protein body pellets and homogenous protein bodies with spherical inclusions are synthesized in aleurone layers during endosperm development (Donhowe and Peterson 1983). Using scanning electron microscopy, the presence of several minerals

such as sulfur, phosphorous, potassium, and magnesium (essential for protein synthesis), along with similar protein composition, were noticed both in the aleurone layer and in starchy endosperm. Optical microscopic studies of transverse section of oat endosperm and flour particles showed a strong adhesion between starch granules and protein bodies. The proportion (less than 3 percent) and consistency of starch granules with storage proteins also determined that is important to know the endosperm hardness of oat (Sikorski 2001).

3.6.5. Fourier transform infrared (FTIR) and Raman spectroscopy

FTIR and Raman spectroscopic methods act as complementary and confirmatory form of analysis to measure the interaction of energy with the molecular bonds in a sample of an unknown material. The FTIR has shown to be best for comparison studies and to monitor the change in secondary structure especially beta structure of denatured oat globulin. Conformational changes of oat globulin fractions during different heat treatment were studied with FTIR. These studies showed progressive changes in the intensity of the alpha-helix, beta-sheet, and beta-turn peaks, suggesting protein denaturation and marked intensity for the formation of aggregated strands (Ma, Rout, and Mock 2001). Raman spectroscopy has been shown to be effective for monitoring confirmation changes in the alpha-helix of oat globulin and the tertiary structure of oat proteins *in situ* (Ma et al. 2000).

3.6.6. Western and Northern blotting

Western blotting is an analytical method for detecting specific proteins in a sample of tissue homogenate or extract (Ida et al. 1996, Burnette 1981). Western blotting studies similarities in disulfide association and molecular weight heterogeneity of globulin protein of oats and rice. When the residual proteins of oat were analyzed with anti-oat 12S globulin using Western blotting, 70–80 percent of the total protein of oat was confirmed as globulin and the recovered glutelins represented less than 10 percent of the total protein (Robert, Nozzolillo, and Altosaar 1985).

Northern blotting is a technique for studying the gene expression by detecting RNA or isolated mRNA in a sample (Alwine, Kemp, and Stark 1977). Northern blot analysis of isolated oat globulin RNAs and their invitro translations showed that there are three major species of poly mRNAs coding, for 18 S, 15 S, and 12S subunits of globulin, respectively. The 18S mRNA is encoded for several 60 kDa polypeptides, indicating that a heterogeneous group of globulin precursors are forming in the initial stages of globulin synthesis that account for 30 percent of the total mRNA in a developing seed and 15S and 12 S mRNAs account for much less (Fabijanski, Matlashewski, and Altosaar 1985)

3.6.7. Light Scattering

Light scattering methods of protein detection are very sensitive can detect or size particles up to $\sim 0.5\ \mu$m. Dynamic light scattering instruments connected with separation methods detect heterogeneity with regard to the molecular weight, composition, and structure of oat albumin and globulin (Runyon et al. 2013). Increases in complexity during heat processing were also elucidated using a MALLS detector combined with AsFIFFF (asymmetrical flow field-flow fractionation) techniques (Runyon et al. 2015). However, light scattering methods are highly sensitive to background contamination, meaning that extra care is required for the preparation of samples.

3.7. Characterization

3.7.1. Amino acid composition

Amino acids (AAs) are the basic building blocks of proteins. Based on the nutritional requirements, AAs are classified into two categories: indispensable and dispensable AAs (Micronutrients, 2005). The nutritional qualities of dietary proteins are determined by the concentration of indispensable amino acids in the protein. Both conventional and modern analytical techniques have been applied for the characterization of AAs of oat and composition (Egorov, Musolyamov, Andersen, et al. 1994, Ewart 1968, Liu et al. 2009, Maruyama et al. 1975, Mustafa et al. 2007, Pomeranz, Robbins, and Briggle 1971, Robert, Nozzolillo, and Altosaar 1985, Runyon et al. 2015).

The detection and quantification of oat AAs using automated AA analyzers was employed by Moore and Stein, who developed the first analyzer in 1951. The basic processes behind all automated analyzers are ion exchange chromatography to separate underivatized and derivatised constituents of AAs, followed by formation of colored products with ninhydrin and detection in the UV-visible region. Robinson et al. (1971) analyzed the AA content of 289 oat samples grown during 1900–1970 in the US and Canada using a hydrolysis method on a Beckman 21 automatic AA analyzer (Pomeranz, Robbins, and Briggle 1971). The values were given for the percentage of protein with maximum and minimum values obtained for individual amino acids. Variability among samples for those nutritionally limiting AAs in oats, such as lysine, glutamine-glutamic acid, and threonine, were also reported. So far, to best of our knowledge, this is the only comprehensive data that is still available for researchers to compare their results (Pomeranz, Robbins, and Briggle 1971).

AA analyzers have been used to analyze husked and naked oats, hand-separated oat groats, and commercially developed oat products (Pomeranz, Youngs, and Robbins 1973);, to perform surface hydrophobicity characterization of hydrophobic/hydrophilic AAs in heated, non-heated, and urea-treated samples (Givens, Davies, and Laverick 2004); and to characterize AA analysis of oat protein isolates (Liu et al. 2009).

Other analytical techniques applicable to characterize proteins and their AA composition are the Edman method of protein degradation, followed by AA characterization using GC and HPLC (Walburg and Larkins 1983). Using ultracentrifugation, the AA composition of oat groats was separated and analyzed and similarities in amino acid composition between cultivars were identified (Burgess et al. 1983). The sulfur-containing AA and the N terminal AAs of hand-separated oat groats and tissues were separated using the Dansyl chloride procedure and characterized by ion-exchange chromatography (Pomeranz, Robbins, and Briggle 1971). Alcohol extraction, followed by a Phenomenex-EZ-Faast amino acid analysis kit, combined with GC, was able to extract and determine 20 amino

acids in less than one hour. Using this method, most common amino acids were found to be alanine, serine, asparagine, and aspartic acid in oats (Mustafa et al. 2007).

3.7.2. Thermal stability

Stability of protein is one of the critical factors for characterizing its use in food systems requiring thermally mediated processes. Oat proteins are thermally stable; in particular, oat globulins have a high thermal denaturation temperature at 110°C. Several techniques have been used to investigate thermal properties oat proteins; these include UV and fluorescence spectrophotometry, Fourier transform infrared (FTIR), Raman spectroscopy, multiage, and quasi-elastic light scattering (Zhao, Mine, and Ma 2004). Differential scanning colorimetry (DSC) is the only technique used to determine the colorimetric effects of protein undergo thermal denaturation (Freire 1995). Thermal stability and thermal denaturation capacity oat globulin were successively analyzed using DSC (Harwalkar and Ma 1987, Ma and Harwalkar 1988). DSC has also been used to analyze stability, as well as modification globulin proteins under various chemicals treatment (SDS, urea, ethyl glycol, and DTT), pH modifications, and stress under different mechanical conditions (Ma and Harwalkar 1988). FTIR was also used to analyze the formation of soluble and insoluble aggregates of oat globulin and their spectral changes during heat treatments (Ma, Rout, and Mock 2001). Asymmetric flow field flow fractionation (AF4)-MALS/UV and refractive indices showed changes in different protein fraction and a progressive decrease in albumin protein with heat treatment (Runyon et al. 2015).

3.7.3. Functional properties

Functional properties of proteins are the inherent nature of a particular protein and their changes in physicochemical attributes when a food undergoes different types of processing, storage, and consumption (Kinsella and Shetty 1979). Both the applicability and potentiality of proteins in a food system depends upon the functionality and its capacity to interact with other components (Damodaran 1997). The extent of changes in functional properties are determined by the AA composition, the type and number of AAs, as well as their structural arrangement in a polypeptide chain (Nakai 1983). Solubility, viscosity, water binding, gelation, cohesion-adhesion, elasticity, emulsification, foaming, fat, and flavor binding are some examples of the functional properties; the most important of these are solubility, emulsions, and foaming. Several modifications can be applied to improve the functionality and nutritional quality of protein, including physical, chemical, and enzymatic; the latter is the most common and simplest of these methods (Panyam and Kilara 1996).

The functional properties of oat proteins have been studied using analytical methods such as trubidometry and dynamic oscillometry for elastomeric properties (Pearce and Kinsella, 1978), gel hardness by the back extrusion method (Gutkoski and El-Dash 1999), and surface hydrophobicity by the fluorescence probe method and gel filtration chromatography (Ma et al., 1998 (Ma and Khanzada 1987a). Trubidometry was used to analyze increases in solubility, emulsifying properties, and fat-binding capacity of the acetyl and succinyl protein concentrate (APC, SPC) of oat proteins (Ma and Wood 1987a, Ponnampalam et al. 1988). Emulsion capacity and emulsion activity index were increased through acetylation and succinylation that was analyzed using trubidometry and dynamic oscillometry (Yong et al. 2004, Mirmoghtadaie, Kadivar, and Shahedi 2009). The amino acid analysis of unmodified acetylated and succinylated oat proteins was conducted using ion exchange chromatography, which showed that the essential amino acid profile of chemically modified proteins was similar to the control. No change in lysine (the most limiting amino acid in oats) has occurred due to acylation and succinylation. Several analytical procedures have been used to determine the water hydration capacity (WHC) (Siu et al. 2002), fat-binding capacity (Ma and Wood 1987a), and foaming capacity and stability (Yasumatsu et al. 1972) of oat proteins.

4. Discussion

This paper reviewed 137 papers, published between 1970 and 2015, that dealt with various analytical methods used for the extraction, separation, detection, and characterization of oat proteins. The review revealed that much of the work carried out is decades old and that very little research was reported between 1990 and 2005 (Table 3). However, interest has been renewed in the use of oat proteins for human consumption, which has led to increased analysis of oat protein fractions and amino acid composition. The purpose of this review was to provide insights into the various analytical methods and their ability to unravel the complexity and nutritional properties of oat protein.

4.1. Extraction conditions and their effectiveness

The review revealed that various methods employed for oat protein extraction suffer harmonization of the type and effect of buffers used for optimization of yield and of the standardized method for removal of unwanted materials during individual protein fractionation. The characterization of oat proteins was started by T.B. Osborne (1901), who used different buffers for the separation of different protein fractions. Osborne's method gave dissimilar proportions of globulin, albumin, prolamins, and glutelins (Osborne solubility fractions) in various studies (Peterson and Smith 1976, Völker 1975, Wieser, Seilmeier, and Belitz 1980b, Peterson 1978, Robert et al. 1983). For the salt-soluble globulin, the reported values range from 12–80 percent (Peterson and Smith 1976, Robert et al. 1983, Peterson, Brinegar, and Webster 1986) and for acid or alkali-soluble glutelins it varies from 5–66 percent (Völker 1975, Wieser, Seilmeier, and Belitz 1980a). These discrepancies are due to a number of factors or errors in sampling, extraction procedures, medium of extraction, cleanup procedure, instrumental analysis, and differences in methods used for calibration/quantification (Wu et al. 1972, Völker 1975, Peterson and Smith 1976, Wieser, Seilmeier, and Belitz 1980a). According to the needs of an optimal extraction buffer with favorable conditions (pH, temperature, ionic strength) harmonization of available extraction methods will then be recommended.

4.2. Separation methods and their efficiency

Ultracentrifugation, chromatography, and electrophoresis are the most widely used means of purifying and separating individual oat proteins. The first two of these are variable

and ranged from low to relatively high agreement between the obtained concentration and complexity. In general, elucidation of complexity for different protein fractions using chromatographic methods are more absolute (Brinegar and Peterson 1982, Walburg and Larkins 1983) than the information obtained by ultra-centrifugation methods (Peterson 1978); see Table 2.

The literature review revealed that different chromatographic techniques have been used to separate individual proteins fractions and the elucidation of their physical, chemical, and structural composition and properties. Among the different chromatographic techniques, HPLC has been the most widely used technique (Table 2). Cation and anion exchange chromatography and gel filtration chromatography have been used; these have also been briefly outlined in this review.

A prominent development in oat protein separation is achieved when HPLC techniques are combined with other separation methods, such as SDS-PAGE or 2D-HPLCs. The combined method relies on the separations of proteins by chromatographic mode before SDS-PAGE that provide better resolution and reproducibility (Pernollet et al. 1989, Wieser and Belitz 1989, Egorov, Musolyamov, Andersen, et al. 1994). In addition, the combined use of analytical methods enables direct analysis of protein fractions, molecular weight determination, and complexity of polypeptides. Using ion exchange chromatography in combination with 2D electrophoresis, the hexametric nature of oat globulin, α, and β chain separation has been elucidated (Peterson 1978). The other chromatographic methods discussed in this review are HPCE capable of separating protein by size, FZCE by charge, isoelectric point and the 2D-HPLC (HPLC combined with HPCE) by charge and size (Lapvetelainen, Bietz, and Huebner 1995, Lookhart and Bean 1995). Another valuable method for oat protein separation is Reverse-phase HPLC, which separates proteins according to surface hydrophobicities. This method appears to be much more effective than conventional chromatographic techniques because it separates proteins based on size and charge differences (Wieser and Belitz 1989).

Combining RP-HPLC with SDS-PAGE has helped identify the polymorphic nature of avenin and its potential use for cultivar identification compared with globulin and albumin (Lookhart and Bean 1995). In general, chromatographic techniques combined with electrophoretic method showed higher reproducibility than applying each technique separately. Therefore, this technique can be used for future routine analyses of oat proteins to identify the complexity at the individual level.

4.3. Detection methods and accuracy

The literature surveyed indicated that the colorimetry and spectroscopy are the most widely used detection techniques for oat protein analysis. Colorimetric studies were mainly used for the analysis of change in functional properties of oat protein under different processing (Ma and Harwalkar 1984), while spectroscopic studies (Raman spectroscopy and Raman- FTIR spectroscoypy) were mainly used for protein confirmation studies (Ma et al. 2000, Ma, Rout, and Mock 2001). Over a certain period of time, most workers used the reverse-phase mode with UV absorbance or flourescence detection. Both methods have several advantages, in that detection occurs at short time period with high sensitivity. However, some disadvantages were observed using UV and flourescence detection. The fluorescence can be affected by the pH of the sample solution, while rate absorption can be influenced by temperature, pH, impurities and contaminants and all these factors can change the absorption. Also, a reduction in flouresect read-out was noticed in spectoscopic analysis of plant protein identification using visible laser excitation due to the presence of some impurities (Wieser and Belitz 1989). In most detection methods, calibration curve analyte concentrations are plotted against instrument response that calculates the concentrations in the sample. The use of internal standards is helpful in terms of accounting for the drifts in instrumental responses and limits of detection. However, only a few reports were found on choice of the internal standard on the comparison of oat protein fractions and their obtained concentration (Ma et al. 2000, Runyon et al. 2013, Runyon et al. 2015).

4.4. Characterization of oat proteins using combined analytical techniques

The literature surveyed indicated that the most of the characterization procedures also exploit the combinations of different analytical techniques based on their key strengths and to overcome the limitations relative to the importance and uniqueness of samples investigated. The high thermal stability and gelation properties of oat globulin preparations have been characterized using differential scanning colorimetry (DSC) combined with SDS-PAGE (Ma, Khanzada, and Harwalkar 1988). When HPLC was combined with DSC, significant differences in heat coagulation of oat globulin compared with other proteins were also noticed (Ma and Khanzada 1987a, Ma and Wood 1987a). Combining DSC and HPLC helped to characterize degradation of oat globulin by alkali treatments, partial denaturation, and alterations in the oligomeric structure (degradation and aggregation) with heat and alkali treatment. The degree of racemization, increase in turbidity, decrease in sulfhydryl content, and distinct changes in physicochemical attributes of globulin were determined by combining different analytical methods (Ma, Harwalkar, and Paquet 1990). When FSCE was combined with RP-HPLC, 480 oat samples were analyzed in a 24 h period, which was faster than any other methods reported to date (Bean and Lookhart 1999). Thus, the combined use of different analytical methods facilitated the characterization of oat proteins and their nutritional importance; this approach will enable researchers to accurately define, quantify, and purify oat proteins.

5. Summary and future directions

The purpose of this literature review was to bring together the different kinds of analytical methods that have been applied to extract, separate, detect, and characterize oat proteins. In presenting this information, we have attempted to compare the different analytical methods and how successful each was in terms of analyzing oat storage proteins and their solubility fractions. However, most of the published studies concentrated on the potential utility of oat proteins for cultivar identification,

in order to improve the nutritional quality and functionality for human food and animal feed. Relatively little attention has been paid to comparing the reliability and potentiality of existing analytical methods for oat protein analysis. The survey revealed that most of the analytical methods need more validation using molecular and biotechnological approaches. Thus, combined use of existing analytical methods couples to current sophisticated techniques in cereal science and technology is inevitable for further method harmonization of oat proteins. This will certainly lead to increased analytical sensitivity.

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