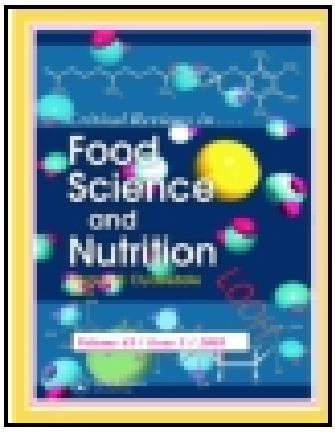

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Speciation of Animal Fat: Needs and Challenges

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Abstract

The use of pork fat is a concern for Muslims and Jews, who for religious reasons avoid consuming anything that is pig-derived. The use of bovine materials, including beef fat, is prohibited in Hinduism and may also pose a risk of carrying the infectious agent for bovine spongiform encephalopathy. Vegetable oils are sometimes adulterated with animal fat or pork fat with beef fat for economic gain. The development of methods to determine the species origin of fat has therefore become a priority due to the complex and global nature of the food trade, which creates opportunities for the fraudulent use of these animal fats as food ingredients. However, determining the species origin of fats in processed foods or composite blends is an arduous task as the adulterant has a composition that is very similar to that of the original fat or oil. This review examines some of the methods that have been developed for fat speciation, including both fat-based and DNA-based methods, their shortcomings, and the need for additional alternatives. Protein-based methods, specifically immunoassays targeting residual proteins in adipose tissue, that are being explored by researchers as a new tool for fat speciation will also be discussed.

Keywords: animal fat, species, protein, DNA, immunoassay

Introduction

Oils and fats derived from both plant or animal sources have long played an important role as an essential nutrient in the human diet and are particularly important in food preparation due to their multiple functional properties. Nutritionally, plant oils tend to be preferred and are more widely used in food production because of the unhealthy fatty acid (FA) profile of animal fats, which have a high saturated FA and low polyunsaturated FA content (Enser, 1995). There is therefore a continued trend towards the use of vegetable oils in food formulations as opposed to the use of animal fats (Orthoefer, 1996). However, because animal fat is cheaper and readily available, food manufacturers in many countries substitute edible animal fat (particularly lard) for vegetable oils as a food ingredient (Aida et al., 2005) or blend lard into edible oil/fat products such as butter and shortening (Sonntag 1982). Adulterating vegetable oils with lard (rendered pig fat), however, is an affront to practitioners of Islam and Judaism, who are forbidden by the dictates of their religion to consume anything derived from pig as spelled out by the halal and kosher dietary laws, respectively. Another issue is that adulterating vegetable oils with tallow (rendered beef fat) may present a health concern as there is a possibility of tallow carrying the agent that causes transmissible spongiform encephalopathy (TSE) (ECSSC, 1999). There are also those who refrain from consuming these animal fats simply for health reasons because of their unhealthy fatty acid profile, which has been implicated in such diseases as cancers, hypercholesterolemia, multiple sclerosis and coronary heart disease.

Despite the predominant use of vegetable oils for food formulation, animal fats such as lard, which has traditionally been used for deep frying many types of food, are still used to some extent because in addition to being economical, they have good stability and functionality, and

their ability to impart unique flavors is considered desirable for some foods (Love, 1996). Current trends in food formulation even suggest that edible animal fats may be enjoying something of a comeback, as the use of vegetable oils as replacements for animal fats is not without its problems and the vegetable oils may not be as beneficial as once thought. For example, there is a growing awareness of the destruction of tropical forest to make way for oil palm plantations, but palm oil is nutritionally not very different from lard as they both have a similar FA profile that lacks significant amounts of the more beneficial omega-3 fatty acids (Bonner, 2012). There is also the issue of the harmful trans fatty acids (TFAs) known to be associated with the hydrogenation of vegetable oils. Animal fats may thus be regaining their prominent role in many food formulations. Thus, to protect consumer interests and ensure fair trade, methods to distinguish the species origin of animal fats are urgently needed to prevent the adulteration of vegetable oils with animals fats and the adulteration of one type of animal fat with another, as in the case of the adulteration of lard with much cheaper tallow (Vaclavik et al., 2011).

Today's complex global food market has increased the potential for such fraudulent activities. There is also an increasing awareness among consumers regarding the ingredients used in the formulation of foods. Thus, efforts to authenticate the species origin of fats have become a priority. However, determining the identity of animal fats in processed foods or composite blends is often difficult as the adulterant may have a composition that is very similar to that of the original fat or oil. The following sections will examine some of the challenges facing those seeking to develop new methods for the speciation of animal fat.

Fat-Based Methods

Fat-based methods rely on subtle differences in the chemical (fatty acid composition and/or their positional distribution on the triacylglycerol [TAG] molecule) or physical (molecular structure and melting/crystallization temperatures) nature of different animal fats to identify their species origin. However, using the fatty acid profile as a means for species identification of animal fat is not straightforward as the fatty acid composition is greatly influenced by the dietary fat intake. For example, there are two major naturally occurring *trans* fatty acids found in ruminant animals (cows, sheep and goats) being conjugated linoleic acid (CLA 18:2 *cis*-9, *trans*-11 and *trans*-10, *cis*-12) and vaccenic acid (VA 18:1, *trans*-11). VA can be metabolized by human to CLA via endogenous synthesis. They are synthesized by some microorganisms occurring in the guts of ruminants through the process of biohydrogenation. However, to date no methods have used the CLA or VA as a suitable marker for ruminant species identification and quantification. This is because CLA is not exclusively found in ruminants as it is also formed in non-ruminants although to a much lesser extent compared to ruminants (Khanal and Dhiman, 2004). Besides, the CLA content in tissues depends on the feeds consumed by the animals during production; CLA contents are greater in pasture-fed cattle compared to grain or silage fed cattle (Ponnampalam et al., 2006). The situation is further compounded by the modern tendency to modify the fatty acid composition of animal tissues, such as by enrichment with omega 3 fatty acids (Wood et al., 2004). This notwithstanding, species-specific differences in the digestion process of dietary fats (Raclot et al., 2001; Sato et al., 1998; Yeaman, 2004) and the different nutrient demands of divergent species, which is ultimately reflected in the composition of the

deposited lipids (Kagawa et al., 1996; Schreiner et al., 2006), have been exploited for species identification of fat. Typically, the fat is removed by saponification, converted to methyl esters, and the fatty acid (FA) pattern analyzed by techniques such as gas chromatography (GC), high performance liquid chromatography (HPLC), or Fourier transform infrared (FIT) or near infrared (NIR) spectroscopy. These techniques are often combined with chemometric techniques such as principal component analysis (PCA) or linear discriminant analysis (LDA) to allow for the recognition of patterns within the large data sets typically generated by the use of such instruments. As the data summarized in Table 1 shows, a number of these fat-based methods have been developed to (i) discriminate lard from other animal fats (particularly tallow), (ii) detect the adulteration of lard with tallow or vice versa, and (iii) detect the adulteration of vegetable oils with animal fat (lard or tallow).

Discriminating lard from other animal fats. Differences in fatty acid methyl esters (FAMEs) have been examined using gas chromatography coupled to time of flight mass spectrometry. PCA has also been utilized to distinguish lard from fat of other species, for example chicken fat, beef tallow, mutton tallow, and cod liver oil in the study by Chin et al. (2009) and from cattle fat, chicken fat and goat fat in another study by Indrasti et al. (2010). A recent study by Nizar et al. (2013) demonstrated the need for PCA, as a direct comparison of fatty acid data obtained by FAMEs analysis with gas chromatography mass spectrometry (GC-MS) was found to be inadequate as a way of determining the species origin of fat from different animals because both lard and chicken fat and beef and mutton fat shared common fatty acid profiles. Subjecting the fatty acid distributional data to PCA allowed the four fats (lard, chicken, beef and mutton) to be clustered into four subclasses. To deal with the inefficiencies involved in

using differences in FAMEs as a means of fat speciation, Nizar et al. (2013) proposed the use of carbon isotope ratios ($\delta^{13}C$) calculated from bulk carbon analyses of these animal fats using elemental analyzer-isotope ratio mass spectrometry (EA-IRMS) as a faster and more effective way to distinguish lard from other fats. Szabó et al. (2007) tried a different approach, conducting fatty acid positional distribution analyses of triacylglycerols (TAG) and 2 monoacylglycerols (2MAG) using gas liquid chromatography as a method of species identification or differentiation of lipid traces from animal sources. Although all the above-mentioned methods may be useful in discerning the species origin of pure fat samples, none have been shown to be useful in detecting adulteration of fat from one species with fat from another. As mentioned in the introductory paragraphs, to protect consumer interests, it is important that the methods developed should not only discriminate among pure fat samples, but also be able to detect the presence of one fat type in another. The next section will look at other methods that have been used to discriminate between pure fat samples from different species, as well as those with the capacity to detect the presence of specific fats in admixtures.

Detecting the adulteration of lard with tallow or vice versa. Two methods, multi-dimensional off-line non-aqueous reversed phase-argentation liquid chromatography coupled to mass spectrometry and direct analysis in real time (DART) ionization coupled to time-of-flight mass spectrometry (TOFMS), have been employed to detect beef tallow in lard (Dugo et al., 2006) and for the discrimination of lard and tallow and their admixtures (Vaclavik et al., 2011), respectively. Both methods rely on differences in the TAG profiles to distinguish lard from tallow. In the case of the method proposed by Dugo et al. (2006), the second step (mass spectrometry) was necessary because mixtures of lard containing 5% and 10% of tallow did not

show significant differences in their chromatographic profiles when analyzed with the first technique (liquid chromatography) alone. Subjecting fractions collected from the liquid chromatography to mass spectrometry enabled the detection of 5% tallow in lard. The second method allowed lard and tallow to be differentiated based on apparent differences in the mass to charge ratio of the TAG fragment ions released by the ionization of TAGs (Vaclavik et al., 2011). This method had a detection limit of 10% (w/w) tallow in lard and 5% (w/w) lard in tallow and compared to Dugo et al. (2006), has the advantage of allowing for the simultaneous detection of lard in tallow and vice versa. It has been reported that there is likely to be an economic incentive for adulteration levels of above 1% of sample weight (Hsieh et al., 1995), so it is therefore desirable for methods for detecting lard in tallow or tallow in lard to have a detection limit of at least 1% (w/w). Thus, the high detection limit of both methods, especially for tallow in lard (10% w/w), limits their usefulness.

Polymorphic differences between fats measured with Raman (Motoyama et al., 2010) and Fourier Transform Infrared (FTIR) (Che Man and Mirghani, 2001) spectroscopy have also been used to detect pork fat in other animal fats. A single Raman band at 1417cm^{-1} that is present in pork fat but absent in beef fat allows pork fat to be distinguished from beef fat (Motoyama et al., 2010). For pork fat in beef fat mixtures, the detection (presence of the band at 1417cm^{-1}) of pork fat was only observable at inclusion levels greater than 50% (w/w). Although this method has the advantage of allowing the fat sample to be analyzed as is without the need for sample preparation, this extremely high detection limit is a major disadvantage. The method proposed by Che Man and Mirghani (2001), on the other hand, had a comparatively low detection limit of 10% (w/w) pork fat in lamb or beef fat. However, their technique was not able to detect pork fat

in chicken fat because of the poor correlation between changes in the spectra due to the addition of pork fat and the blend ratio. In addition, this method involved the use of adipose tissues sourced from various parts of slaughtered pigs, cows, lamb and chicken that were collected from a local market. Further studies may therefore be necessary to validate the effectiveness of this method against commercial fat samples, as tallow and lard that have been produced through the normal rendering process will have been subjected to manufacturing processes such as severe heat treatment that may upset the spectral pattern.

Detection of the adulteration of vegetable oils with animal fat (lard or tallow). As mentioned previously, the adulteration of vegetable oil with animal fats is a more common occurrence. Accordingly, many of the fat-based methods for fat speciation have focused on detecting the adulteration of vegetable oils with animal fat. Methods based on the so-called "electronic nose" (zNose^{TM}) (Che Man et al., 2005), differential scanning calorimetry (DSC) (Marikkar et al. 2002), and Fourier transform infrared (FTIR) spectroscopy combined with chemometrics involving partial least square (PLS) and discriminant analysis (DA) (Rohman et al., 2011), have all been developed to detect animal fat, particularly lard, in various vegetable oils. The zNose^{TM} chromatogram for refined, bleached and deodorized (RBD) palm olein is distinctly different from that of lard due to the presence of less volatile compounds in RBD palm olein (which has been refined, bleached and deodorized) compared to the strong and characteristic swine odor, and subtle differences in the aroma pattern of unadulterated (0%) RBD palm olein compared to RBD palm olein adulterated with 1% (w/w) lard were discernible (Che Man et al., 2005). However, because the peak area changes were relatively subtle for the samples containing 1% (w/w) lard, the detection limit for this method was deemed to be 3% (w/w) lard,

at which point the peak areas were dramatically different from those for unadulterated RBD palm olein. The use of the electronic nose to detect lard adulteration in RBD palm olein is not only fast, but also does not require any sample pre-treatment or chemicals for the analysis. However, it may not be effective in situations where the volatile compounds have been masked by processing, as for example in the case of deodorized lard. The scope of this method may need to be better defined.

Animal fats typically contain TAG molecules that have relatively high amounts of saturation compared to canola oil, a vegetable oil with less saturation in its TAG molecules, and this has been used as a basis for detecting the presence of animal fats in canola oil using cooling and heating differential scanning calorimetry (DSC) thermograms (Marikkar et al., 2002). Amounts as low as 2% (w/w) tallow and 8% (w/w) lard could be detected in canola oil using this method. The addition of chicken fat, however, did not produce any changes in the peak pattern of unadulterated canola oil, thereby making detection of chicken fat in canola oil impossible with this approach. Although the authors mentioned that the adulteration peak corresponding to lard had a characteristic shape and position, on first glance this looks identical to the peak pattern for tallow in lard, and it would thus require an extremely high level of expertise to distinguish between lard in canola oil and tallow in canola oil adulterations. The high detection limit (8% w/w) of lard in canola oil was also noted by the authors, who highlighted the need to use other techniques to detect samples containing lower levels.

The study by Rohman et al. (2011) exploited a minor difference in peak height between lard and vegetable oils at 1117 and 1097 cm^{-1} using FTIR spectroscopy combined with

chemometrics [partial least squares (PLS) model and discriminant analysis (DA)] to analyze lard adulteration in vegetable oils . They reported that their PLS calibration models made it possible for quantification of lard in vegetable oils such that levels as low as 1% (v/v) of lard in some vegetable oils could be detected. However, these vegetable oils were not specified. In addition, DA allowed classification of pure vegetable oils from those adulterated with lard. Unlike the two methods described above, both of which clearly define their scope in terms of the detection of lard in RBD palm olein (Che Man et al., 2005) and lard and tallow in canola oil (Marikkar et al., 2002), the same cannot be said of this method (Rohman et al., 2011).

DNA-Based Methods

DNA molecules are now becoming the target for species identification in foods because of their relatively high stability and presence in most biological tissues and are highly specific (Mafra et al., 2008). With these DNA-based methods, mitochondrial DNA is generally the target as it has several advantages over nuclear DNA (Kocher et al., 1989; Rastogi et al., 2007; Unseld et al., 1995). The specific amplification of a fragment of DNA by means of a polymerase chain reaction (PCR), with subsequent fragment size verification based on gel electrophoresis, is the simplest DNA-based strategy for species identification. Species-specific variations such as RFLP (restriction fragment length polymorphism) PCR, the analysis of single strand conformation polymorphism (SSCP) PCR, sequencing of fragments, and the simultaneous amplification of two or more fragments with different primer pairs (multiplex PCR) have been developed for species identification. PCR and its PCR-RFLP variant in particular have been used for the speciation of edible animal fats, as summarized in Table 2. Specifics of these DNA-based methods are highlighted in the next sections.

RFLP-PCR analysis of a conserved region in the mitochondria cytochrome b (mt cyt b) gene (Aida et al., 2005), species-specific PCR analysis of a conserved region in the mitochondrial 12S ribosomal RNA (rRNA) (Aida et al., 2011) and porcine-specific primers of pork D-loop mitochondria DNA (mtDNA) (Montiel-Sosa et al., 2000), have all been utilized for the species identification of pork meat and fat. In the first two studies (Aida et al., 2005; Aida et al., 2011), the fat samples used for the study were trimmed from sheep, cow, chicken and pig meats that were bought from a local meat market. Animal fats that are used commercially are subjected to severe heat-processing in the form of rendering that may affect the quality and quantity of DNA present in the sample. Hence, these two methods cannot be guaranteed to be effective against commercial animal fat samples unless they have been tested. In a later study, Aida et al. (2007) applied their previously described PCR-RFLP technique (Aida et al., 2005) for the detection of pig derivatives in sausages (made from chicken, beef, pork, and non-labeled meat), casings (non-labeled), bread and biscuits, where the method was deemed ineffective against heat-treated samples. No genomic DNA was detected from the casing samples, which the authors ascribed to the casings being made of synthetic fibrous material, and the genomic DNA extracted from biscuit and bread was of low yield and poor quality. As the bread samples were made with vegetable fats, which are plant-based products, there was no mitochondria cytochrome b gene present since the cytochrome b primer can only be used to amplify homologous segments of mitochondrial DNA from animal species. In the case of the biscuits, which were either commercially produced or home-made with 1%, 5% and 50% (w/w) of the fat content consisting of lard, the lack of PCR amplification of the target gene was attributed to either the degradation of the DNA by the high processing temperatures (details of which were

not provided) or a lack of sufficient target DNA or DNA, which may have been contaminated by inhibitors such as organic and phenolic compounds. However, given that some of the biscuit samples were formulated with as much as 50% lard, insufficient target DNA is unlikely to be the reason. DNA degradation by the thermal process, as postulated by the authors, seems more credible as the study involved the use of commercial lard samples produced through rendering, involving heat-treatment, coupled with further heat-treatment by way of baking. In the other study by Montiel-Sosa et al. (2000), pork meat and fat was detectable in mixtures of pork (meat or fat) in beef at inclusion levels down to 5% (w/w). Although the authors asserted that this method is useful even when heat-treated samples are involved, this is debatable as little or no information regarding the time-temperature regiment applied to the products was provided. Compared to fat-based methods, less effort has gone into the development of DNA-based methods for fat speciation, probably because researchers believe adipose tissue is not a significant source of DNA. Instead, most research in this area has focused on the development of DNA-based methods for meat speciation that could also be applied to fat speciation, as in the examples above.

Challenges of Fat- and DNA-based Methods

A number of fat-based methods using various instruments, either singly or in tandem, have been employed to determine the species origin of fat (Table 1). Besides their individual limitations, pointed out above, although most of these fat-based methods may be useful they are laborious, and require long testing times, an experienced analyst, and the use of expensive equipment. They also generally need to be combined with complex multivariate statistical analysis techniques.

Because of the shortcomings of fat-based methods, the DNA-based methods discussed above and summarized in Table 2 have been employed as an alternative approach for the species identification of animal fat as they are sensitive, faster and easier to perform than the fat -based methods. Although this article has focused on the DNA-based methods developed specifically for animal fat speciation, several other DNA-based methods have been utilized for the species identification of meat (see the review by Kumar et al., 2013) and could equally be applied for fat speciation, given that DNA-based methods are not tissue-specific. However, although DNA-based methods are useful and are considered to have the most potential as a method for fat speciation by some researchers, their success depends on the amount and quality of the DNA extracted from the sample. Several food processes have a negative influence on the accessibility and extraction of appropriate DNA material for PCR, thus rendering DNA-based methods ineffective in certain situations. For example, DNA can be degraded by high-temperature food processes either directly (Bellorini et al., 2005) or indirectly through the action of radicals created by the Maillard products generated during the thermal processing (Hiramoto et al., 1994). DNA may also be degraded during food processes such as hydrolysis (both enzymatic and chemical) and mechanical treatment (shear-forces) (Jacobsen and Greiner, 2002). Typically, DNA is not detectable in highly heat-processed food products, hydrolyzed products, and highly purified products such as refined oils (Kuiper, 1999). As noted earlier in the discussion of the study by Aida et al. (2007), even at inclusion levels of as much as 50% (w/w), the amount of pig fat DNA extracted from biscuit samples was of low yield and quality, rendering the lard undetectable. Prado et al. (2004) also reported that high temperature processes such as heating at

133^{o}C under pressurized autoclave conditions resulted in severe DNA fragmentation and consequently poor DNA extractability.

Another problem is that most of these methods, whether fat or DNA-based, have focused on the speciation of fats as raw ingredients. Thus, although these methods can be used to identify the species origin of raw fat samples, this is not a viable approach when the animal fats are present as ingredients in processed foods. As seen above, in the few instances where the methods were tested on processed samples, they did not produce useful results. Methods for fat speciation have been developed for the authentication of fats used in feed formulation as a BSE control measure (Abbas et al., 2009; Bellorini et al., 2005), for forensic purposes (Kagawa et al., 1996; Moawad et al., 2009), and as an indirect approach for meat speciation (Chernukha, 2011). However, although these methods could equally be applied to the speciation of animal fats, they suffer from the same shortcomings as the above-mentioned methods, being laborious, applicable only to raw fat samples, and/or unable to distinguish beef fat from lamb fat. Consequently, not only is it necessary that these methods be validated against different food matrices formulated with animal fat, but also that other methods using different markers be developed to complement the existing fat-based and DNA-based methods.

Potential of Imuunoassay as a Means to Distinguish Fat Species

Although protein-based methods have been used over the years as analytical tools for meat species identification they have not generally been applied to fat speciation, probably because adipose tissue is not thought to contain sufficient amounts of protein. Immunoassays are widely accepted by regulatory bodies as a quick and sensitive method for screening and

monitoring a number of substances in food and agricultural products because they do not require major investments in equipment, are easy to perform, need only small quantities of test sample and immunoreagents, and have the capacity to be utilized for large-scale screening. They can also be formatted into user-friendly kits that can be used by producers, regulators and even individual consumers. We therefore examined the potential utility of immunoassays as a protein-based method to detect the very low levels of residual protein in adipose tissue as a means to distinguish the species origin of fat based on the premise that adipose tissue does indeed contain sufficient amounts of protein to permit the detection of these protein traces in fat using immunoassays for fat speciation.

Adipose tissue contains sufficient amounts of protein. Fat trimmings constitute the raw material for rendering processing. The beef fat trimming typically produced by a USDA plant consists of 14 to 16% fat, 60 to 64% moisture, and 22 to 24% protein (Franco and Swanson, 1996). The objective of the rendering process is to separate the proteins and moisture from the fat in order to obtain as pure a form of fat as possible. However, irrespective of the processing method, the final product (fat) invariably contains some amount of insoluble impurities, which are harmless filterable materials that are insoluble in petroleum ether. These insoluble impurities can be either meat and bone particles that remain after the rendering operation, or foreign matter such as sand and or metal particles that have contaminated the product post-processing. The bulk (85%) of the insoluble impurities have been estimated to be proteinaceous (APAG, 2003). Even in the case of tallow, which is typically subjected to more stringent processing to remove as many of the contaminating proteins as possible to prevent the spread of BSE, the level of insoluble impurities in tallow decanted from large holding tanks can be as high as 0.5% (APAG,

2003). However, in both the EU and the US, tallow is subjected to further purification processes as a BSE-preventive measure to reduce the level of insoluble impurities to a maximum content of 0.15% (w/w), as per the recommendations of the World Organization for Animal Health (OIE, 2007).

Adipose tissue inherently secretes different types of proteins that play important roles in homeostasis and metabolism through their autocrine, paracrine, and endocrine effects. These secreted proteins include hormones, cytokines, extracellular matrix proteins, neural proteins, and proteins that are involved in the formation of blood vessels and capillaries and in the metabolism of lipid and glucose (Rosenow et al., 2010). The term adipokine has been suggested to describe all proteins secreted from any type of adipocyte (Trayhurn et al., 2011). Changes in the secretion of these proteins during adipocyte differentiation lead to differences in their circulation profiles, which invariably result in regional differences in the adipocyte proteins isolated from different adipose locations. Gondret et al. (2008) utilized 2-dimensional fluorescent differential gel electrophoresis to examine protein extracts from adipocytes obtained from muscle and other fat locations (inter-muscular, subcutaneous, and perirenal) of pig muscle, noticing differences in proteins expressed in muscle-derived adipocytes and those derived from other adipose locations. Only three proteins, including the known skeletal muscle protein α-actin, were expressed in greater amounts in muscle-derived adipocytes compared with adipose tissue from the other locations. Other researchers have also demonstrated the differential expression of skeletal-muscle-associated proteins such as tropomyosin-1 α chain, tropomyosin β chain, myosin regulatory light chain 2, myosin light chain 3, and parvalbumin α, troponin I (TnI), and collagen in adipocyte tissues isolated from various species (Ahmed et al., 2010; Tong et al., 2005; Wang

et al., 2006; Yang et al., 2010a; Yang et al., 2010b). These regional differences in protein expression in adipocytes obtained from different adipose locations will not affect the total protein present in a rendered fat tissue as lard or tallow, however, as the fat trimmings used as raw material are obtained from different locations on the carcass. The findings of these studies clearly show that animal fat contains a significant amount of protein as expressed proteins and meat particle contaminants in the form of residual insoluble impurities that can be assayed using sensitive immunoassay techniques.

Detection of protein traces in fat for fat speciation using immunoassays. Bellorini et al. (2005) compared the abilities of four different methods, namely Fourier transform infrared spectroscopy (FTIR) applied to fat samples; gas chromatography coupled with mass spectrometry (GC-MS) to determine fatty acid profiles; PCR for bovine-specific DNA; and immunoassay ruminant test kit (*AgriScreen for Ruminant in Feed STRIP TEST*, Neogen Corporation, Lansing, MI, USA), to differentiate between the sources of fat used in animal feed. Although the ruminant test kit was originally developed to detect ruminant meat and bone meal in animal feed as a preventive strategy for BSE, it was included in the study to reveal whether the residual insoluble impurities in rendered fat contain adequate amounts of proteins to permit detection by sensitive immunoassays. This ruminant-specific test kit proved useful for detecting low concentrations (5% w/w) of tallow in a mixture of fat (tallow in lard or chicken fat) prepared by the rendering industry even when samples had been sterilized at temperatures >133^{o}C. The PCR method was also able to detect 5% w/w of tallow in admixtures of fat that had been sterilized at temperatures >133^{o}C, although neither the PCR nor the immunoassay was able to detect “premier jus” tallow, which is the highest quality standard of fat and has an extremely low

concentration of protein (Bellorini et al., 2005). The FTIR and GC-MS techniques, however, were only effective in detecting the pure fat samples. It is worth noting that the mAbs used in the manufacture of the *AgriScreen for Ruminant in Feed STRIP TEST,* which targets the skeletal muscle protein troponin I (TnI), were originally developed in our laboratory to detect rendered muscle tissue in animal feedstuff (Chen et al., 2002). Recently, by using a simple improved sample preparation method in conjunction with this commercial lateral flow strip immunoassay (later renamed *Reveal for Ruminant in Feed*), a detection levels as low as 2% (w/w) bovine fat in pork fat, 1% (w/w) bovine fat in porcine MBM, and 0.5% (w/w) bovine fat in soy meal mixtures can be achieved (data not published yet). Another pork-specific immunoassay (sandwich ELISA) that was previously developed in our laboratory to differentiate pork meat from meat of other species using two mAbs that bind cooperatively to skeletal muscle TnI (Liu et al., 2006) has also been found to be effective in discriminating between pork fat and fat from other species (beef and chicken), as well as for detecting low levels of raw and cooked pork fat (0.3% to 2%, w/w, depending on the mixture and heat treatment) spiked in beef or chicken fat or meat (data not published). These results not only support the potential application of immunoassay techniques for determining species content of fat adipose tissue, but also suggest that adipose tissue contains sufficient quantities of muscle proteins to enable their use as a bovine species marker for fat detection in an immunoassay.

Conclusions

As the research discussed here shows, both fat-based and DNA-based techniques have been found to be useful for the species identification of animal fat. However, in addition to their

other shortcomings, these methods have focused almost exclusively on the speciation of raw fat. Thus, although the usefulness of these methods for identifying the species origin of raw fat samples can be vouched for, the same cannot be said in situations where these animal fats are present in processed foods. As noted above, in the few instances where the methods were tested on processed samples, the methods were found to be inadequate for fat speciation. Instead, protein-based methods such as those based on immunoassays that detect protein traces in the residual insoluble impurities (RIIs) of fat samples may serve as an alternative and complementary tool for the rapid species identification of the species origin of fat. Immunoassays have the unique advantage of being conveniently formatted into user-friendly kits that can be used even by individual consumers in the home. However, as with other methods, immunoassays have their drawbacks, as shown in the study by Bellorini et al. (2005), in that they may not be effective against highly refined animal fats because of the extremely low protein concentration in their RIIs. Since DNA-based techniques are also ineffective against highly refined fats, fat-based techniques may be the method of choice when such samples are involved.

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Table 1: Fat-based methods for edible fat speciation

Objective	Technique	Basis of specieation	Reference	Detection limit
Detect lard blended with chicken, lamb and cow fats	Fourier transform infrared (FTIR) spectroscopy	Spectral bands	Che Man and Mirghani (2001)	1. 10% w/w pork fat in lamb fat 2. 10% w/w pork fat in beef fat 3. Pork fat in chicken fat was undetectable
Monitor the presence of tallow, lard and chicken fat in canola oil	Differential Scanning Calorimetry (DSC)	Cooling and heating thermograms	Marikkar et al. (2002)	1. 2% w/w tallow in canola oil 2. 8% w/w lard in canola oil 3. Chicken fat in canola oil was undetectable
Detect lard adulteration in	Electronic nose	Aroma pattern (chromatogram)	Che Man et al. (2005)	1% w/w lard in RBD palm olein

refined bleached and deodorant (RBD) palm olein Detect tallow in lard	HPLC in combination with atmospheric pressure chemical ionization mass spectrometry (APCI-MS)	Fatty acid distribution along the glycerol backbone	Dugo et al. (2006)	5% w/w tallow in lard
Species identification of fat from red deer, moose, wild boar, farmed pig, badger, rabbit, and goose	Gas liquid chromatography	Fatty acid distribution along the glycerol backbone	Szabo et al. (2007)	Not determined
Distinguish lard from chicken	Fast comprehensive	Fatty acid methyl ester (FAME)	Chin et al. (2009)	Not determined

fat, beef fat, mutton fat, and cod liver oil	two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (fast GC x GC-TOFMS)	profiles		
Detect the difference between lard and three other animal fats (cattle fat, chicken fat and goat fat)	Comprehensive gas chromatography hyphenated with time-of-flight mass spectrometry	Fatty acid methyl ester (FAME) profiles	Indrasti et al. (2010)	Not determined
Discriminate between pork lard and beef tallow and their admixtures	Direct analysis in real time (DART) ionization-mass spectrometry combined with	Triacylglycerol profiles	Vaclavik et al. (2011)	1. 10% w/w tallow in lard 2. 5% w/w lard in tallow

	chemometric tools			
Detect lard adulteration in some vegetable oils	Fourier transform infrared (FTIR) spectroscopy combined with chemometrics	Spectral pattern	Rohman et al. (2011)	1% v/v lard in vegetable oils
Differentiate amongst lard, chicken fat, beef fat and mutton fat	Gas Chromatography Mass Spectrometry (GC-MS) and Elemental Analyzer-Isotope Ratio Mass Spectrometry (EA-IRMS)	Carbon isotope ratios ($\delta^{13}C$)	Nizar et al. (2013)	Not determined
Distinguish between pork fat and beef fat	Raman spectroscopy	Polymorphic features of fats	Motoyama et al. (2010)	50% w/w pork fat in beef fat

Table 2: DNA-based methods for edible fat speciation

Objective	Technique	Basis of Speciation	Referenece	Detection limit
Distinguish meat and fat of pig from those of other species (sheep, cow, and chicken)	PCR-RFLP	Species-specific difference in a conserved region in the mitochondria cytochrome b gene	Aida et al. (2005)	Not determined
Detect pig derivatives (pork and lard) in food products	PCR-RFLP	Species-specific difference in a conserved region in the mitochondria cytochrome b gene	Aida et al. (2007)	Homemade biscuits formulated with 1% and 50% of lard could not be detected
Distinguish meat and fat of pig from those of other species (sheep, cow, and chicken)	PCR	Species-specific difference in a conserved region in the mitochondria 12S ribosomal RNA	Aida et al. (2011)	Not determined
	PCR			5% w/w pork fat

Detect pork meat and fat in meat products	Published species differences in nucleotide sequences of the D-loop region of mitochondria DNA	Montiel-Sosa et al. (2000)	in beef

