CHROMATOGRAPHIC METHODS TO ANALYZE GEOMETRICAL AND POSITIONAL ISOMERS OF FATTY ACIDS: A REVIEW

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ABSTRACT

Problems in analyzing fatty acids are to obtain good resolution of geometrical and positional isomers. In GCMS analysis, common derivative of fatty acids such as fatty acids methyl ester (FAME) will not give mass spectra that indicative of the true structure of fatty acids, thus structure elucidation of fatty acids will be misled. This review describes chromatographic methods to obtain good resolution of geometrical, positional isomer of fatty acids and to determine the double bond position in fatty acids. Methods that are described are gas chromatography (GC), gas chromatography mass spectrometry (GCMS), thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) impregnated with silver ion. Derivatization methods to determine double bond position of fatty acids are also described. Applications, advantages and drawbacks of every method are discussed.

Keywords: Positional and geometrical isomer; gas chromatography; mass spectrometry; silver ion chromatography

INTRODUCTION

Analysis of fatty acids structure is significant in fields such as biochemistry and food science. For instance, trans-11-octadecenoic acid might not pose danger to health (Molkentin and Precht, 1995). This is in contrast to its positional isomer, the trans-9-octadecenoic acids which are known to give deleterious effect. However, geometrical isomer of trans-9-octadecenoic acids which is the cis-9-octadecenoic acids, acts like any other cis isomer, which do not pose danger to health. Chatgilialoglu et al. (2002) reported that changes of geometry and position of double bonds can effect the physical properties of membrane bilayer. So analysis of double bond position and configuration in fatty acids may be of special interest as negative metabolic activities might originate from certain isomers. Numerous studies reported that fatty acids composition, positional and geometrical isomers in cooking oil are altered after thermally treated. (Romero, 2000; Karabulut, 2003; Liu, 2007; Kandhro, 2008; Tsuzuki, 2008; Alededunya, 2009; Bansal, 2009). Shifting of double bond position and configuration can occur if the fatty acids is treated at high temperature. So isomeric distribution of fatty acids analysis may give great impact on nutritional studies besides food qualities. However, analysis of fatty acids structure is complex due to wide range of isomers that can occur from certain fatty acids. The main problems are to determine double bond position of fatty acids along the carbon chain, besides to obtain good resolution of fatty acids geometrical and positional isomer in gas chromatography analysis. This review describes methods to analyze fatty
acids positional and geometrical isomer, and to determine double bond position of fatty acids, along the carbon chain.

**ANALYSIS OF ISOMERS**

Usually fatty acids isomers are analyzed by gas liquid chromatography (GC) or gas chromatography mass spectrometry (GCMS)

**Gas Chromatography**

Gas liquid chromatography has been the methods of choice for half a century. This is due to properties of GC which is sensitive, rapid and precise, give good reproducible analysis. GC also offers convenience and relatively low in cost compared to other methods. Fatty acids are separated by the interaction with mobile and stationary phases in a column, and detected by a detector. Common detector for GC is flame ionization detector (FID) which can detect fatty acids down to picogram level. Results are interpreted from peaks in chromatograms. Good resolutions are shown by sharp and symmetric peaks. Resolutions in GC are affected by polarity of stationary phase and column length. Better resolution and separation is achieved when packed column is substituted by capillary column, and when stationary phase polarity and column length is increased. Lots of research conducted showed that stationary phase polarity and column length give great impact to resolution of fatty acids isomers. Tavella et al. (2000) reported that on highly polar CP Sil 88 column, 50 meter long column gave good separation of positional and configurational isomer where column in 30 meter long only separated positional isomers. Liu et al. (2007) tested four columns to analyze trans fatty acids in hydrogenated oil. Using DB-1 60 meter column, nine cis isomers could be separated, for Innowax 30 meter and 60 meter, both could separate 6 trans and nine cis isomers, but the later with longer elution time, HP 88, 100 meter in length could separate nine cis and eight trans isomers. Molkentin and Precht (1997) obtained better resolution using HP 88, 100 meter than 50 meter column. Figure 1 shows the chromatogram of this analysis. The chromatograms show that more isomers are resolved using longer column. Research conducted by Wolff and Bayard (1995) discovered that doubling the length of CP Sil 88 column from 50 meter to 100 meter greatly improved resolution of fatty acids isomers, however both column could not separate trans-13 and trans-14 isomer. Juanida (2002) succeeded in separating trans-13 and trans-14 isomer using BPX 70 column. Other studies that achieved good separation using longer column are Glew et al. (2006) and Golay et al. (2006). Ledoux et al. (2000) succeeded in separating cis and trans isomer using SP 2560 and CP Sil 88 column. Even with long, highly polar column, GC condition need to be optimized. Wolff and Bayard (1995) didn’t achieve good separation using SP 2560, 100 meter compared to HP Sil 88 column, 50 meter because the condition using SP 2560 is not optimized.

Non volatile sample is problematic in GC analysis so fatty acids need to be esterified to render it to be more volatile. However, if fatty acids are not esterified completely, resolution of minor isomers will be badly affected. The present of non saponifiable materials also will effect GC resolution. So prior GC analysis, fatty acids need to esterified and purified, thus increasing analysis time. Minor isomers tend to be overlapped behind major isomers, so pre fractionation steps is needed before subsequent GC analysis, rendering more analysis time. In GC FID, fatty acids are identified by comparing retention
time against standard and due to limited fatty acids standard and complexity of fatty acids composition, it is always difficult to identify some peaks with conventional FID detector.

Figure 1: Chromatograms of fatty acids methyl ester with A: in 50 meter column and B: in 100 meter column (Molkanten, 1995).

**Gas Chromatography Mass Spectrometry**

GCMS is gas chromatographic (GC) technique combined to mass spectrometer (MS) detector. This is the method where peak identification problem, faced by GC FID is solved. GCMS is a powerful tool in identifying peaks. In GCMS, fatty acids are ionized, forming charged fragments with certain mass. Fatty acids are identified based on mass to charge ratio, and compared with a library of known mass spectra which is stored on computer database. Kandhro et al. (2008) tested both GCFID and GCMS in analysis of trans fatty acids in margarine. Due to production of new artificial fatty acids during the manufacturing process, it was difficult to identify peaks of new fatty acids using GCFID, they obtained more accurate peak identification using GCMS. The usefulness of GCMS depends on its resolution which is the ability to distinguish two particles of different masses. So even GCMS can give good peak identification, overlapping of isomers still occur in GCMS, so pre fractionation is needed prior GCMS analysis for better resolution. Pre fractionation is discussed further in the next section. Main difficulty of GCMS is determination of position and configuration of double bonds. Usually fatty acids is analyzed as fatty acids methyl ester (FAME) in GCMS, however FAME spectra is not indicative of double bond position. This is because double bond migration occurs during ionization of FAME, so fatty acids need to be derivatized where double bonds are fix to prevent migration. The fixing of double bond will be discussed further in derivatization section.

**PRE FRACTIONATION BY SILVER ION CHROMATOGRAPHY**

Pre fractionation is to simplify mixture of fatty acids, concentrate minor components for better resolution of GC and GCMS analysis. Silver ion chromatography is said to be powerful in fractionating fatty acids prior subsequent GC or GCMS analysis. Separation of fatty acids isomer is based on complexation between double bonds in fatty acids and silver
The complex is charge transfer type and the formation of complex is reversible. Since the complexation occurs at double bonds, fatty acids are separated according to unsaturation degree. Saturated fatty acids do not form complex with silver ion, so are eluted quicker than unsaturated fatty acids. Polyunsaturated fatty acids are held strongly by silver ion. The complexation between cis isomer is more stable than trans isomer. Stability of complex is decrease by increase of chain length. In fatty acids with two double bonds, the further the two double bonds are separated, the complex is more stable. For example double bonds between C6 and C10 are retained stronger than C9 and C12, thus, double bonds in methylene interrupted system are held stronger than conjugated double bonds.

Types of chromatography that applied silver ion chromatography are thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

**Silver Ion Thin Layer Chromatography**

In silver ion TLC, fatty acids are separated according to number and configuration of double bonds. Figure 2 shows typical fatty acids separation in silver ion TLC. Methods of impregnation of silver ion into silica gel and solvent choice are important. In most cases, the plates are dipped in 5% to 20% AgNO₃ in water or acetonitrile, then air dried, activated at high temperature, developed in a solvent system, then spots are visualized by spraying with dichlorofluorescein. Destaillant et al. (2006) used 5% of AgNO₃ to prepare plate while Wilson and Sargent (2001) utilized 10%, Molkentin and Precht (1997) applied 20% solution of AgNO₃ in water (w/v) for 20 minutes, air drying, activated at 120°C for 30 minutes, developed in heptane: diethyl ether (90:10), sprayed by 0.2% dichlorofluorescein in isopropanol. Using the system, ten trans isomers and nine cis isomers were separated. Instead of hexane, Golay et al. (2006) utilized heptane in the solvent system.

![Figure 2: Fatty acids separated according to unsaturation degree and position of double bonds (Dobson, 1995)](image-url)
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At the end of TLC run, silica gel were scraped off, FAME extracted using methanol in NaCl or diethyl ether, then analyzed by GC or GCMS. Sometimes glass plates are replaced by plastic sheets that can be cut using scissor, silica gel no longer scraped from the sheets, but specific areas can be cut from the sheet, and plunged into solvent solution for extraction (Ledoux et al., 2000). Destaillant et al. (2006) compared single method of GC and silver ion TLC combined with GC method. Separation by GC alone was good, but better separation was achieved when the two methods were combined. On 100 meter GC column, 95% isomers were separated, however 100% isomers were separated if fatty acids were fractionated by silver ion TLC. So for analysis of isomeric distribution, GC or GCMS as a stand alone method is good, but excellent result can be achieved is pre fractionation steps utilizing silver ion TLC is utilized. Silver ion TLC is easiest to use, cheapest, require only typical lab equipment. It is also can be operated by unskilled analyst (Wilson and Sargent, 2001). However, it is time consuming, laborious and no possibility for automation (Ledoux et al., 2000).

Silver Ion High Performance Liquid Chromatography

Silver ion HPLC is to supply fatty acids fractions of same number and configuration of double bonds for further GC analysis. Figure 3 shows how fatty acids are separated according to unsaturation degree. Saturated, cis monoenoic, trans monoenoic and polyenoic are separated in different peaks. Factors effecting elution and resolution in silver ion HPLC are methods of impregnating silver ion into column, mobile phase composition and column temperature.

Figure 3: Analysis of FAME by silver ion HPLC. Fraction A, B, C, D is saturated, trans monounsaturated, cis monounsaturated and diunsaturated fatty acids respectively (Gollay, 2006)
Column preparation

Three methods are available in preparing column. First method is packing standard column with silica impregnated with known amounts of silver ion. However this method requires skills and practice. Second method is to convert standard cation exchange column into silver ion form. This method led to the availability of commercial column, the Chromspher Lipid. Utilization of silver ion HPLC increased rapidly with the introduction of commercial column. Third method is to add silver ion solution (Aqueous AgNO3 solution or silver perchlorate in isopropanol/ methanol) in mobile phase. This method is corrosive for the column, so it will deteriorate rapidly and selectivity of separation is changed. The problem with column is to obtain stable and reproducible stationary phase (Destailant et al., 2007), with a controlled silver ion content and reasonable working life (Ledoux et al., 2000). AgNO3 may bleed from the column thus limiting working life. Adlof and List (2004) achieved column reproducibility even after 70 samples over four weeks duration.

Mobile phase composition

Mobile phase composition is important as it will determine interaction between analyte, silver ion and mobile phase. The choice of mobile phase composition traditionally is based on TLC experience. Solvent system for silver ion HPLC normally are toluene based, hexane based and dichloromethane based. Hexane-acetonitrile is widely used, especially for commercial column (Damyanova, 2009). Usually, 1.0% to 1.5% acetonitrile in hexane is utilized. Adlof et al. (1995) in analyzing isomers in hydrogenated vegetable oil, utilized acetonitrile in hexane as solvent, however trans-8 and trans-9 could not be separated. In 1997, Adlof reported that improved resolution was achieved with increased content of acetonitrile in hexane. Momchilova et al. (1997) also reported the same case where small changes in acetonitrile content will influence retention and resolution, so acetonitrile is suggested to take part in interaction between silver ion and double bonds. (Momchilova et al., 1997). Momchilova (1998) studied the effect of mobile phase composition on the resolution of isomers. Clear resolution was achieved using hexane: dichloromethane; acetonitrile (60: 40: 0.2) in 22 minutes. Dichloromethane was added to overcome the problem of acetonitrile insolubility in hexane. Insolubility of acetonitrile causes difficulty in applying gradient elution (Damyanova, 2009). 1.5% of dichloromethane will help solubility of acetonitrile. Besides improving solubility, dichloromethane also improved retention. With the increase of dichloromethane level, retention will be decreased. (Momchilova, 1997). Research conducted by Adlof and List (2004) reported no evidence of acetonitrile insolubility at the level of 1.0% to 1.5%.

Temperature

In contrast to other form of chromatography, lower temperature will result in shorter elution time in silver ion chromatography. Adlof and List (2004) studied the effect of column temperature on elution time. At lower temperature, components were eluted quicker. This is probably because acetonitrile-silver ion complex may be temperature induced charged. The complex is exothermic, less stable at high temperature thus allowing more interaction between silver ion and double bond, so retention is increased (Adlof and List, 2004). Adlof (2007) also studied the limitation of low temperature application in silver ion HPLC. The research discovered that low temperature application were limited to
-25°C to 70°C. This may be because, at lower temperature than the range given, acetonitrile is not soluble in hexane, solubility factor may effect the amount of sample and acetonitrile that can interact with silver ion. Temperature effect only detected in hexane base solution, not in chlorinated or hydrocarbon based solvent system.

**Advantages and Drawbacks of Silver Ion HPLC**

The advantages of silver ion HPLC are chromatograms are easier to interpret than reverse phase HPLC. Analysis time is short, about 15 to 20 minutes running time, complete separation of cis and trans can be achieved. Silver ion HPLC can be good alternative to silver ion TLC where application of silver ion TLC is messier. In HPLC, the collection of fractions can be automated where in TLC, fractions need to be scraped from plates (Ledoux, 2000). The drawbacks of silver ion HPLC is the preparing of column and reproducibility. However with the availability of commercial pre coated column might solve the problem.

**DERIVATIZATION TECHNIQUES TO DETERMINE POSITION OF DOUBLE BONDS**

After fractionation, fatty acids will be analyzed by GCMS. Usually fatty acids are analyzed by GCMS as fatty acids methyl ester (FAME). However mass spectra of FAME are not indicative of structure. MS indicates number of double bonds, but the position is unknown. This is because, during ionization process, double bond are also ionized, result in migration of double bonds along the chain. So other derivatization techniques that can fix double bonds, preventing from migration is required. Examples are the formation of dimethyl disulphide (DMDS) adducts and formation of trimethylsilyl (TMS) ether derivatives. Cleavage of components occur at the derivatized double bonds, producing diagnostic ion that can give information about the position of double bonds. However these two techniques are limited to monounsaturated fatty acids, not applicable to polyunsaturated fatty acids (Dobson and Christie, 1996). Types of derivatization techniques that are applicable to polyunsaturated fatty acids are picolinyl esters and 4,4-dimethyloxazoline (Ledoux et al., 2000). These two types of derivatives are nitrogen containing derivatives. As a result of the preferred ionization at the nitrogenous function, the ionization of unsaturated systems in the hydrocarbon chain is suppressed, resulting in a reduced tendency of bond migration along the hydrocarbon chain (Spitzer, 1996). Whenever a double bonds or functional group occur in a chain, cleavage will occur at these sites, producing diagnostic ions. Mejanelle et al. (2002) utilized both FAME and DMOX in elucidating structure of fatty acids marine flagellate. DMOX derivatives gave better result than FAME derivative in elucidating the structure.

**4,4-dimethyloxazoline (DMOX)**

DMOX are simple to prepare compared to picolinyl ester. Preparation usually needs high temperature. Fatty acids need to be hydrolyzed first then reacted with 2-amino-2-methyl-1-propanol in a vial at 180°C for 2 h in a nitrogen atmosphere. Other modes of preparation is reaction at room temperature, preparation directly from FAME mixture without hydrolysis and directly from lipid samples (Spitzer, 1996). Mass spectra of DMOX is better than picolinyl ester in giving information about structure of polyunsaturated fatty acids (Dobson and Christie, 1996). DMOX derivatives is just slightly more volatile than
FAME, so both resolutions are comparable. DMOX derivatives are prone to hydrolysis during storage. Juanida (2001) converted FAME to DMOX after fractionated by SI HPLC and before GC analysis.

**Picolinyl Ester**

The preparation of picolinyl ester require formation of free fatty acids, so fatty acids need to be hydrolyzed prior derivatization. Typical derivatization method is to dissolve fatty acids in thionyl chloride, then reacted with 3-hydroxymethylpyridine in acetonitrile. The reaction is sensitive to moisture, so care must be taken to ensure dryness during preparation. Although mass spectra of DMOX is said to be better in giving information about structure of polyunsaturated fatty acids, picolinyl ester in which the double bonds are reacted with deuterium atoms, can be great value in analyzing polyunsaturated fatty acids (Dobson and Christie, 1996). The major disadvantages of the frequently used picolinyl ester and pyrrolidide derivatives are their low volatility and the loss in GC resolution in comparison to the fatty acids methyl esters (Spitzer, 1996). Both picolinyl ester and DMOX derivatives are powerful in elucidating structure of fatty acids, however sometimes the spectras can lead to many possible structure.

**Other Types of Derivatives**

If only information of monoenoic is required, it is better to convert fatty acids to dimethyl disulphide adducts because this method is satisfactory. The formation of this reagents require only single step reaction and single reagents. Methyl ester are dissolved in dimethyl disulfide with a trace of iodine. Deuteriation is also a technique for locating double bonds. The formation of this derivative requires deuterium gas. It is valuable for characterizing the cyclic fatty acids, enabling location of double bonds both in the aliphatic chain and in the ring structures (Christie, 1998)

**CONCLUSION**

To obtain good result in analysis of positional and configurational isomers, fatty acids mixture need to be fractionated into simpler mixture. Excellent separation and fractionation can be achieved utilizing silver ion chromatography. Prior analysis by GC or GCMS, fatty acids need to be derivatized to derivatives that are indicative of structural features such as picolinyl ester and DMOX. Excellent resolution can be achieved by GC or GCMS if highly polar and long capillary column is utilized. GCMS is the most powerful tool in identification of fatty acids. Excellent methods in analyzing fatty acids positional and geometrical isomers will be a great help in research of food qualities, nutritional and biochemical studies.

**ACKNOWLEDGEMENT**

Authors are thankful and greatly acknowledge the financial support from Research Management Centre, Universiti Malaysia Pahang
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