Isolation of Pure Individual Fatty Acids from Chicken Skin Using Supercritical CO₂ Extractor or Cooling Centrifuge

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Abstract: Chicken skin -a poultry meat industries waste- has been used in this work as a source for the production of pure free fatty acids. Chicken skin fat was extracted using dry rendering method. Physical and chemical parameters of that fat were determined. Also, its fatty acids composition has been identified by GC-MS after its esterification as oleic, palmitic, linoleic, stearic, myristic, lauric, linolenic, behenic, arachidonic, arachidic, palmitoleic, and paullinic acids, and others as traces. The extracted fat was then hydrolyzed into mixture of free fatty acids and glycerol, the free fatty acid mixture was separated, then it was cooled in order to separate saturated and unsaturated fatty acids from each other. Oleic, Palmitic, Linoleic and Stearic Acids were extracted individually in pure form using supercritical CO₂ extractor. Moreover, oleic, linoleic, palmitoleic, linolenic, and paullinic acids were extracted individually in pure form using cooling centrifuge sigma 3-18KS. All of the separated individual fatty acids were confirmed according to their melting point, GC-MS after esterification, elemental analysis and mass spectrometry (ms) of the corresponding methyl ester in order to detect the corresponding molecular ion peak. Therefore, these new two methods could afford the very expensive pure fatty acids with a low cast.

Key words: fatty acids, hydrolysis, extraction, supercritical CO₂ extractor, cooling centrifuge

1 Introduction

Chicken is one of the most popular meat sources all around the world¹, where, there is no religious prohibition against chicken compared to other meats². Chicken skin has been found to form about 11.7 to 15.2% from the total chicken carcass weight³. Consequently, considerable amount of chicken skin has been afforded during chicken carcass portioning⁴, causing environmental pollution⁵,⁶ and loss of a very cheap sources of lipids⁷. On the other hand, chicken skins are well known by their low content of saturated fatty acids and cholesterol and high content of unsaturated fatty acids⁸, thus it’s likely to be used as a source of healthy fat. Also, chicken skins are currently being utilized for production of value added products⁹,¹⁰, e.g. biodiesel⁵,¹¹, biogas, cosmetics¹² and as a vital source for gelatin¹³ and collagen¹⁴. The pure individual fatty acids are very expensive chemical compounds which play a very important role in chemical and pharmaceutical industries, on the other hand all pervious works were aimed to identify the fatty acid composition of chicken skin considered as waste to value and potential source of these molecules to exploit, thus, this work aimed to isolate pure individual fatty acids from this mater by using two new inexpensive methods for their production.

2 Experiment

2.1 Instruments

2.1.1 All melting points were measured on an Electrotherma™ 1A9300 Beginning Ending Recording Model for Pharmacopeia Requirements.

2.1.2 Capillary gas chromatograph (HP 6890) was used for the qualitative and quantitative determinations of fatty acids of the extracted fat samples and reported in relative area percentages.

2.1.3 Supercritical CO₂ instrument used in the present study was applied, Separations, Inc., Allentown, USA, model no.7071.
2.1.4 Cooling centrifuge Sigma 3-18KS instrument was applied in the present study.

2.1.5 The NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer. $^1$H spectra were run at 300 MHz. Chemical shifts are quoted in $\delta$ and were related to that of the solvents.

2.1.6 Mass spectra were recorded on a Shimadzu GC MS-QP 1000 EX mass spectrometer at 70 eV. Elemental analyses were carried out at the Micro-analytical Center of Cairo University.

2.2 Material and reagents

The chicken skin was obtained as a byproduct from Shahd poultry farm and was chilled in ice while transporting to the laboratory. Skin was washed, dried with a towel and weighed for further use.

All solvents and reagents were analytical grade, and were supplied from Merck (Darmstadt, Germany). All experiments and analytical determinations were performed at least in duplicate.

2.3 Chicken skin fat extraction

Chicken skin (1 kg) was minced, lipids were extracted from fatty tissues by dry rendering method at $90^\circ$ C for 3 h. After cooling to $50^\circ$ C, the fats were filtered through Whatman No.1 filter paper. Then it kept in a brown bottle at $5^\circ$ C until analysis.

2.4 Quality parameters

Refractive index, acidity, peroxide value, iodine value and saponification value of the extracted fats were determined according to A.O.A.C.

2.5 Fatty acids composition

The fatty acids composition of the extracted fat was determined by GC-MS after their esterification. Fatty acids were esterified into their corresponding methyl esters by shaking a solution of oil (0.1 g) in heptane (2 mL) with methanolic potassium hydroxide solution (0.2 mL, 2 N). The fatty acid methyl esters were identified using a gas chromatograph. Nitrogen flow rate was 0.6 mL/min, hydrogen and air-flow rates were 45 and 450 mL/min, respectively. The oven temperature was isothermally heated to $195^\circ$ C. The injector and the detector temperatures were $230^\circ$ C and $250^\circ$ C, respectively. Fatty acid methyl esters were identified by comparing their retention times with known fatty acid standard mixture. Peak areas were automatically computed by an integrator.

2.6 Oil hydrolysis.

A fat molecule is an ester of glycerol with three fatty acids molecules. Thus, it could be hydrolyzed with water at high temperature and pressure to produce fatty acids mixture and glycerol. Thus, the extracted chicken skin fat was hydrolyzed with distilled water (2 L) in high pressure reactor at $250^\circ$ C and 2 MPa, then, the reaction was left to cool to $35^\circ$ C, where the reaction mixture was separated into two layers. The upper layer contained the fatty acids while the lower one contained water, glycerol and traces of non fatty materials. The upper layer was then separated, dried over anhydrous sodium sulfate, filtered off and weighted.

2.7 Confirmation of oil hydrolysis

2.7.1 By thin layer chromatographic analysis

Oil hydrolysis was confirmed by thin layer chromatographic analysis, where, plates (20 × 20) were coated with a slurry of silica gel (60 g) in water (15 g/hg), left to dry, then it was activated at $110^\circ$ C for 1.0 h. Standard spot of known fatty acid and other spots of oil (individually) were spotted on the activated thin layer plates, at the base line (2 cm from the bottom). The fatty acids mixture of the hydrolyzed fat was also spotted on the same line. The developing solvent consisted of $n$-hexane, diethyl ether and acetic acid at a volumetric ratio 80: 20: 1, respectively. The developing jar was lined on three sides with filter paper wetted with the same developing solvent. The plates were developed till the solvent reached the front line (15 cm from the start line). The spots of different components separated by TLC were then visualized by iodine vapor. The fatty acids were considered formed when the withdrawn sample showed only one spot with no tail, and with rate of flow similar to that of the known fatty acid spot but not the oil spot.

2.7.2 By $^1$H NMR spectrum

The fatty acids mixture of the hydrolyzed fat was also confirmed by $^1$H NMR spectrum where signal at $\delta 10.06$ was observed which indicates the presence of carboxylic hydrogen of the free fatty acids.

2.8 Fractionation of extracted chicken skin fatty acid mixture

Fatty acid mixture of the extracted chicken skin fat was fractionated into liquid and solid fractions. Then each fraction was weighted.

2.9 Individual extraction of fatty acids by using supercritical CO$_2$ extractor and by cooling centrifuge

Oleic and linoleic acids were extracted individually from the liquid fraction using supercritical CO$_2$ extractor at (30.0 MPa, 313 K) and (27.0 MPa, 333 K) respectively. While palmitic and stearic acids were extracted individually from the solid one using supercritical CO$_2$ extractor at (35.0 MPa, 328 K) and (30.0 MPa, 333 K) respectively. The obtained fatty acids were confirmed according to their elemental
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3 Results and Discussion

3.1 Fat extraction from chicken skin

Chicken skin contains fats, proteins, minerals and some vitamins.\(^\text{20}\) So dry rendering method\(^\text{3}\) was used for lipids extraction from chicken skin (1 kg) and afforded 332.01 g oil which is corresponding to 33.20%.

3.2 Quality parameters

Physical and chemical properties of the extracted chicken skin fat were determined according to A.O.A.C\(^\text{16}\), and the results are listed in Table 1.

3.3 Fatty acids composition

Chicken skin fat that has been thought as waste, was found to be a valuable source of fatty acids, those possess analysis, melting points, GC mass and mass spectra\(\text{ms}\) of their methyl esters which detect the corresponding molecular ion peaks.

On the other hand, the liquid fraction was cooled to \(-11.20^\circ\text{C}\) with centrifugation at 9000-1200 rpm, using cooling centrifuge, then getting rid of all the melted products. Temperature was then raised up to the melting point of linoleic acid\((-11.00^\circ\text{C}\) with centrifugation at the same speed, the melted product was collected.

Subsequently, temperature was raised up to \(-5.2^\circ\text{C}\) with centrifugation at the same rpm, and disposing of all the melted products. Temperature was afterwards increased to the melting point of oleic acid\((-5.00^\circ\text{C}\) with centrifugation at 9000-1200 rpm, and collecting of the melted product.

Thereafter, temperature was raised up to 0.30\(^\circ\text{C}\) with centrifugation at the same speed and disposing all the melted products, followed by increasing temperature up to the melting point of Palmitoleic acid\(0.50^\circ\text{C}\) with centrifugation at the same rpm, and collecting of the melted product.

Afterwards, temperature was increased to 12.00\(^\circ\text{C}\) with centrifugation at the same speed and getting rid of all the melted products. Then temperature was increasing up to the melting point of Oleic acid\(13.00^\circ\text{C}\) with centrifugation at the same rpm, and the melted product was collected.

Temperature was then raised up to 13.2\(^\circ\text{C}\) with centrifugation at the same speed and disposing of all the melted products. Then temperature was increasing up to the melting point of Palmitic acid\(13.40^\circ\text{C}\) with centrifugation at the same rpm, and collecting of the melted product.

All of the collected products were identified individually using G-C mass, and was also confirmed according to their elemental analysis, melting points, GC mass and mass spectra\(\text{ms}\) of their methyl esters which detect the corresponding molecular ion peaks.

| Table 1 | Physicochemical properties of chicken skin. |
| --- | --- |
| Parameter | Chicken skin |
| Refractive index at 25\(^\circ\text{C}\) | 1.46 |
| Acid value (as oleic acid) | 0.7 |
| Peroxide value (meq/kg oil) | 1.9 |
| Iodine value (Hanus) | 78 |
| Saponification number | 201 |
| Unsaponifiable matter (%) | 0.27 |

Data are expressed as mean ± SD values given represent means of three determinations.

Table 2 displays chicken skin fatty acids profile according to its GC-MS scan\(^\text{17}\), in which twelve fatty acids were identified. The predominant fatty acids were oleic acid\((\text{C}_{18:1})\), palmitic acid\((\text{C}_{16:0})\), linoleic acid\((\text{C}_{18:2})\) and stearic acid\((\text{C}_{18:0})\) respectively, so chicken skin could be considered as a very rich source of oleic acid. On the other hand, the unsaturated fatty acids were oleic acid\((\text{C}_{18:1})\), linoleic acid\((\text{C}_{18:2})\), palmitoleic acid\((\text{C}_{16:1})\), linolenic acid\((\text{C}_{18:3})\), behenic acid\((\text{C}_{22:1})\), arachidonic acid\((\text{C}_{20:4})\) and paullinic acid\((\text{C}_{22:1})\). Moreover, the unsaturated fatty acids concentration was nearly twice as the saturated fatty acids, thus, increases the benefits of chicken skin fat.

3.4 Oil hydrolysis

Hydrolysis of chicken skin fat (332.01 g, 33.2% of skin)
according to method described by Hanaa et al.\textsuperscript{18,19}. Afforded a free fatty acids mixture (305.33 g, 30.53% of skin) in addition to water, glycerol and unsaponifiable materials. Formation of fatty acids was confirmed according to TLC analysis, \textsuperscript{1}H NMR spectrum that reflect a carboxylic hydrogen signal at \textit{δ} 10.06. Moreover, the obtained glycerol (26.76 g, 2.68% of skin) indicates complete chicken skin fat hydrolysis.

3.5 Fractionation of extracted chicken skin fatty acids mixture

The free fatty acids mixture (305.33 g, 30.53% of skin) contains saturated and unsaturated fatty acids, according to their physical properties, all saturated fatty acids are solids while all unsaturated fatty acids are liquids at 7°C, thus, saturated and unsaturated fatty acids could be separated from each other by cooling at 7°C at which the saturated fatty acids are solidified and precipitated while the unsaturated fatty acids keep in their liquid state\textsuperscript{9,19}. So, they could be easily separated from each other by decantation. Then each fraction was weighted where the solid and liquid were found to be 104.55 g and 199.6 g, respectively.

3.6 Individual extraction of fatty acids by using supercritical CO\textsubscript{2} extractor and by using cooling centrifuge

The difficult of fatty acids mixture separation is mainly attributed to the tiny difference in their polarity. As carbon dioxide polarity in its supercritical condition is highly affected by temperature and pressure, it could be used as an ideal solvent for fatty acid mixture separation.

Thus, the predominant fatty acids of the fatty acids mixture (305.33 g) of chicken skin fat (332.01 g) were extracted individually in pure form using supercritical CO\textsubscript{2} extractor. Oleic and linoleic acids were extracted individually from the liquid fraction at (30.0 MPa, 313 K), (27.0 MPa, 333 K) respectively, and with yield percentage equal to 99.05 and 99.44 respectively. While palmitic and stearic acids were extracted individually from the solid fraction at (35.0 MPa, 328 K), and (30.0 MPa, 333 K) respectively, and the corresponding yield percentages were equal to 98.12 and 98.65 respectively. All the extracted fatty acids were confirmed according to their melting point, GC mass and mass spectra (m/z) of their methyl esters which detect the corresponding molecular ion peaks, and chemical analysis as described in Table 3.

As each chemical compound has its own melting point which is related to its chemical structure, mixture of free fatty acids could be separated into individual pure fatty acids according to their melting point.

At $-11.20^\circ$C all compounds of melting point lower or equal to this point are going to be melt, but not for those of higher melting point. Thus, getting rid of those melted compounds means getting rid of all fatty acids of melting point lower or equal to $-11.20^\circ$C. As linoleic acid melting point is $-11.00^\circ$C, gradual increases of temperature up to $-11.00^\circ$C cause melting of linolenic acid and any other compound of melting point between $-11.20^\circ$C and $-11.00^\circ$C, but due to the fatty acids composition, there is no other compound at this melting point. Thus, the melted compound at this temperature is only linolenic acid and its yield was 92.5%.

Then, temperature raising up to $-5.20^\circ$C causes melting of all compounds of melting point between $-11.00^\circ$C and $-5.20^\circ$C, and elimination of those melted products means getting rid of all fatty acids of melting point in this range.

Whereas melting point of linoleic acid is $-5.00^\circ$C, temperature increases up to $-5.00^\circ$C cause melting of linoleic acid and any other compound of melting point between $-5.20^\circ$C and $-5.00^\circ$C, but due to the fatty acids composition, there is no other compound of melting point at this melting range. Thus, the melted compound at this temperature is only linoleic acid and its yield was found to be 93.4%. In the same way, palmitoleic, oleic and paluillinc acids were isolated individually as in Table 4. Each isolated fatty acid was confirmed individually according to its G-C mass analysis as shown in Table 4 and according to its elemental analysis and mass spectra (m/z) of its methyl esters which detect the corresponding molecular ion peak as described in Table 5.

### Table 3  Yield, melting points and chemical analysis of the fatty acids extracted with supercritical CO\textsubscript{2} extractor.

| Property | Fatty acid | Oleic acid $C_{18}H_{34}O_2$ | Palmitic acid $C_{16}H_{32}O_2$ | Linoleic acid $C_{18}H_{30}O_2$ | Stearic acid $C_{18}H_{36}O_2$ |
|----------|------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| M. wt (g mol$^{-1}$) | 282.47 | 256.43 | 280.45 | 284.48 |
| Methyl ester molecular ion peak (m/z) | 296(M$^+$) | 270(M$^+$) | 294(M$^+$) | 298(M$^+$) |
| Chemical analysis | | | | |
| Theoretical | C % | 76.54 | 74.94 | 74.09 | 76.0 |
| O % | 12.13 | 12.58 | 11.50 | 12.76 |
| H % | 11.33 | 12.48 | 11.41 | 11.25 |
| Experimental | C % | 76.49 | 74.86 | 74.15 | 76.29 |
| H % | 12.16 | 12.62 | 11.12 | 12.59 |

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Table 4 Separated fatty acids according to their melting points using cooling centrifuge.

| Temperature °C | Melted product | The separated fatty acid |
|----------------|----------------|-------------------------|
|                | Wt (g)         | Yield %                 |
| −11.2          | 0.035          | −                       |
| −11.0          | 1.38           | 92.5                    |
| −5.2           | 0.001          | −                       |
| −5.0           | 48.45          | 93.4                    |
| 0.3            | 0.004          | −                       |
| 0.5            | 9.58           | 92.3                    |
| 12.8           | 0.042          | −                       |
| 13.0           | 123.65         | 93.1                    |
| 13.2           | 0.00           | −                       |
| 13.4           | 0.72           | 90.4                    |

Table 5 Yield, melting points and chemical analysis of the fatty acids extracted by cooling centrifuge.

| property                        | Fatty acid | Paullinic acid C20H38O2 | Oleic acid C18H34O2 | Palmitoleic acid C16H30O2 | Linoleic acid C18H32O2 | Linolenic acid C18H30O2 |
|---------------------------------|------------|-------------------------|---------------------|--------------------------|------------------------|-------------------------|
| M. wt (g mol⁻¹)                 | 310.52     | 282.47                  | 254.41              | 280.45                   | 278.44                 |
| Methyl ester molecular ion peak (m/z) | 321(M⁺) | 296(M⁺)                  | 267(M⁺)              | 293(M⁺)                  | 291(M⁺)                 |
| Chemical analysis               |            |                         |                     |                          |                        |
| Theoretical C %                 | 77.36      | 76.54                   | 75.54               | 74.09                    | 77.65                  |
| Theoretical H %                 | 12.33      | 12.13                   | 11.89               | 11.50                    | 10.86                  |
| Theoretical O %                 | 10.31      | 11.33                   | 12.58               | 11.41                    | 11.49                  |
| experimental C %                | 77.41      | 76.49                   | 75.49               | 74.15                    | 77.69                  |
| experimental H %                | 12.27      | 12.16                   | 12.62               | 11.12                    | 10.82                  |

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