Quantitation of Underivatized Omega-3 and Omega-6 Fatty Acids in Foods by HPLC and Charged Aerosol Detection

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Abstract

The omega fatty acids are a group of compounds that include essential n-3 (omega-3, e.g., α-linolenic acid [ALA]), n-6 (omega-6, e.g., linoleic and arachidonic acids), and nonessential n-9, (omega-9, e.g., oleic and erucic acids) analytes. The omega-3 fatty acids, which also include eicosapentanoic acid (EPA) and docosahexanoic acid (DHA), are required for normal growth. Their consumption is purported to have a number of health benefits: e.g., cancer prevention, cardiovascular disease prevention, and improved immune function. Although both omega-3 and -6 fatty acids can give rise to eicosanoid-signaling molecules (prostaglandins, prostacyclins, thromboxanes, and leukotrienes), the omega-6 eicosanoids are generally pro-inflammatory and may play a role in cardiovascular disease, high blood pressure, and arthritis. It appears that the amounts and balance of omega fatty acids in a person's diet affect their eicosanoid-controlled functions. A proper balance of omega fatty acids in the diet is important.

Traditionally, omega fatty acids are measured using gas chromatography (GC). For foods, analytes are extracted from the samples prior to hydrolysis to release the fatty acids from their triglycerides, then converted to their volatile methyl esters prior to analysis by GC. This approach is tedious, time-consuming, and the high temperatures can affect polyunsaturated fatty acid stability. The Thermo Scientific Dionex Corona charged aerosol detector provides a univeral mass-based approach that is sensitive, has a wide dynamic range, and has a major advantage in that all nonvolative analytes give similar response independent of chemical structure. No derivatization is required, and unlike UV detection, the analyte does not need to contain a chromophore. Presented here is a simple and direct high-performance liquid chromatography and charged aerosol detection (HPLC-charged-aerosol-detector) method that can be used to measure omega-3, -6 and -9 fatty acids in traditional and commercially produced meat, fish, and oils, as well as over-the-counter supplements.

Introduction

The common determination of omega lipids in foods comprises several steps, including extraction, hydroysis, and derivitization for measurement by GC. GC works well as a standard analytical tool for these determinations but requires alteration of the sample, and it can also adversely affect temperature-sensitive functional groups on specialized lipids. HPLC with ultraviolet detection requires use of low wavelengths, which limits solvent selection and increases the likelihood of matrix interference.

Shown here is a reversed-phase HPLC method for the determination of omega fatty acids in oil/food samples using a dual-gradient method and charged aerosol detection. This combination enables determination of many fatty acids in a single analysis, and without the sample derivatization that is required for GC analysis.

Several fatty acids were analyzed, including six omega-3 fatty acids (stearidonic acid [SDA], eicosapentanoic acid [EPA], α-linolenic acid [ALA], docosahexanoic acid [DHA], docosapentanoic acid [DPA], eicosatrienoic acid [ETA]), five omega-6 fatty acids (γ-linolenic acid [GLA], arachidonic acid [Arach.], linoleic acid [LLA], adrenic acid, and eicosadienioic acid [EDA]), and two omega-9 fatty acids (oleic and erucic acids). An omega-7 fatty acid, 9E,14Z-conjugated linoleic acid (CLA), was also included due to its cited importance as a nutrient.[1,2]

Charged aerosol detection is mass sensitive and can be added to HPLC or ultra HPLC (UHPLC) platforms. The detector provides the most consistent response for all nonvolatile and some semivolatile analytes of all HPLC detection techniques.[3] It works by charging particles as shown in Figure 1, and is not dependent on light scattering, which has large variability and generally lower sensitivity.

Charged aerosol detection has been successfully used to characterize lipids of all classification,[4] including phospholipids (reversed phase[5] and normal phase[6,7]), acylglycerides, phytosterols, free fatty acids, and free fatty alcohols. This method complements the free fatty acids method, using higher specificity for these analytes and a Thermo Scientific Acclaim C30 reversed-phase column.

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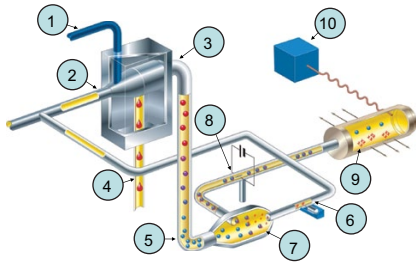
FIGURE 1. Schematic of the Corona™ charged aerosol detector flow paths.



1. Liquid eluent enters from the HPLC system
2. Pneumatic nebulization occurs
3. Small droplets enter the drying tube
4. Large drops exit to drain
5. Dried particles enter the mixing chamber
6. Gas stream passes over corona needle
7. Charged gas collides with particles and transfers charge
8. High mobility species are removed
9. Remaining charged particles measured with a very sensitive electrometer
10. Signal is transferred to chromatographic data software

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Method

Experimental Conditions

HPLC System:	Thermo Scientific Dionex UltiMate 3000 RSLC Dual Gradient
Mobile Phase A:	Water/formic acid/mobile phase B (900:3.6:100)
Mobile Phase B:	Acetone/acetonitrile/tetrahydrofuran/ formic acid (675:225:100:4)
Column:	Acclaim™ C30, 250 × 3 mm, 3 µm
Column Temp.:	30 °C
Flow Rate, Eluent Gradient Pump:	1 mL/min
Flow Rate, Inverse Gradient Pump:	1 mL/min

Eluent Gradient Pump		*Inverse Gradient Pump*	
Time (min)	*% B*	*Time (min)*	*% B*
0.00	0.0	0.00	100.0
1.00	60.0	1.10	100.0
13.00	70.0	2.10	40.0
22.00	95.0	14.10	30.0
24.00	95.0	23.10	5.0
24.00	0.0	25.10	5.0
29.00	0.0	25.10	100.0
		30.10	100.0

Injection Volume:	2.00 µL
Detector:	Corona ultra RS charged aerosol detector
Corona Filter:	High
Corona Nebulizer Temp.:	15 °C
Total Run Time:	30.1 min

The system is configured so that the analytical pump provides the eluent through the column, and the second pump adds the solvents in a manner that is inverse of the column gradient. In this manner, the composition of the eluent entering the charged aerosol detector is maintained at a constant percent organic to minimize changes between relative response factors of the different analytes. It was determined that the void time for the analytical stream was 1.10 minutes greater than that for the inverse-gradient stream, and this time was added to the inverse-gradient pump program, as shown in the table above. This configuration reduces the relative response differences between analytes caused by the changing percent organic in the column eluent.

Standard Preparation

All standards were dissolved and diluted to 2500 µg/mL in alcohol, and diluted serially to a concentration of 6.25 µg/mL.

Sample Preparation

All solid fat samples (50–100 mg) were extracted in 1.2 mL methanol/chloroform (1:1) for 15 min using vortex mixing. Extracts were then centrifuged to remove solids, and 1.0 mL of extract was added to 4 mL of isopropanol/water (3:2) and 1 mL of 5 M KOH.

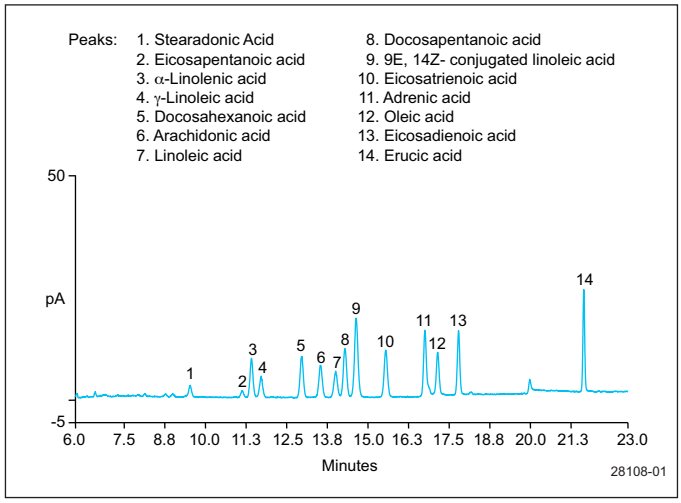
All liquid oil samples (50 µL aliquot) were dissolved/dispersed in 5 mL isopropanol/water (3:2) and 1 mL of 5 M KOH.

All samples were heated in an 80 °C water bath for 1 h with occasional stirring. After samples were cooled, a 500 µL aliquot was removed and 25 µL of formic acid was added to neutralize the sample.

Results and Discussion

A chromatogram of the 14 standards (2500 ng on column [o.c.]) is shown in Figure 2.

FIGURE 2. HPLC chromatogram of 14 omega-free fatty acids.



Peak retention times in min were found to be: SDA 11.8, EPA 13.7, ALA 14.0, GLA 14.4, DHA 15.8, Arach. 16.4, LLA 16.7, DPA 17.0, CLA 17.2, ETA 17.9, adrenic 18.9, oleic 19.2, EDA 19.7, and erucic 23.1 minutes.

Use of the inverse gradient improved the consistency of response across the analytes. The relative spread of responses between the inverse-gradient method, and the gradient-only method decreased by 42%, calculated by ([inverse-gradient max–min] – [gradient-only max–min]/[gradient-only max–min]). This indicates that the use of the inverse gradient can provide a benefit to quantitation when unknown peaks are present in a sample chromatogram, providing for improved quantitation estimates.

It was later discovered that a few of the omega fatty acid standard materials had degraded during the course of developing this method. This is the likely cause of the wide range of responses seen in the calibration curves, shown in Figures 3 and 4. In using a fresh standard for DHA, the response was found to be similar to that of ALA. The use of 10 mg/L of butylated hydroxyanisole (BHA) in the standard and sample solutions may preserve the dissolved analytes for a longer period of time without affecting the chromatography.

Calibration curves, using the inverse gradient conditions data, were fit using inverted second-order polynomials resulting in correlation coefficients, r^2 >0.9995. Triplicate analyses provided peak area reproducibility with an RSD <7% for all amounts ≥25 ng o.c.

FIGURE 3. Calibration curves for SDA, EPA, ALA, DHA, ETA, and CLA from 12.5–5000 ng o.c.

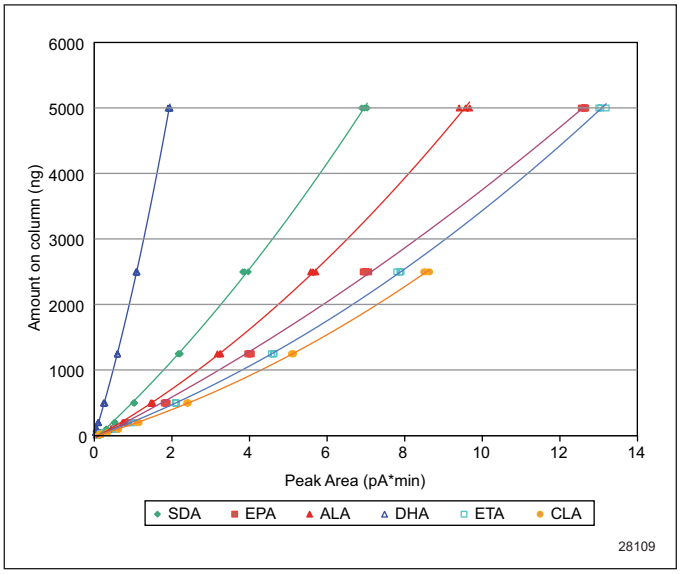
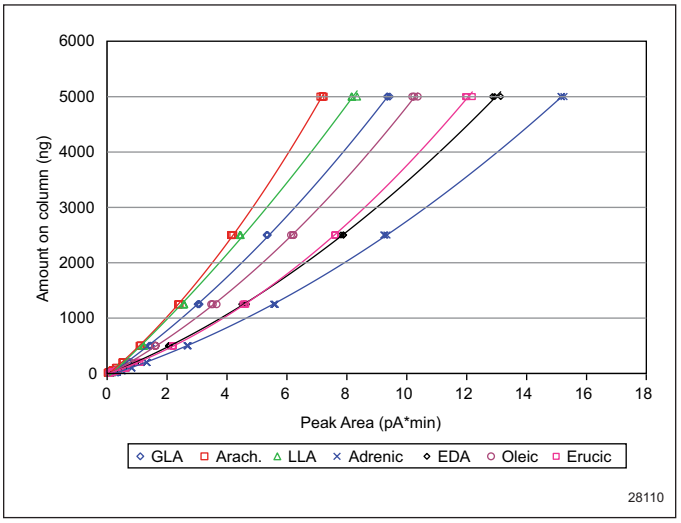


FIGURE 4. Calibration curves for GLA, arach., LLA, adrenic, EDA, oleic, and erucic acids from 12.5–5000 ng o.c.



Sensitivity was determined through sequential dilutions and with a signal-to-noise (S/N) ratio of 3 for the limit of detection (LOD) and 10 for the limit of quantitation (LOQ). These values are presented in Table 1.

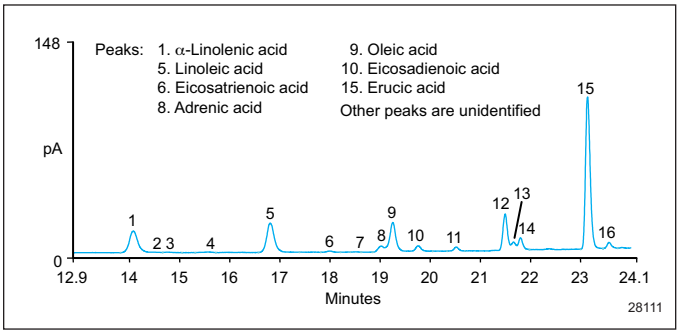
Table 1. LOD and LOQ Values for Omega-Free Fatty Acids by HPLC		
Analyte	**LOD (ng o.c.)**	**LOQ (ng o.c.)**
SDA	9.7	32.5
EPA	11.5	38.5
ALA	13.2	43.9
GLA	13.4	44.6
DHA*	15.0	45.0
Arach.	21.4	71.4
LLA	10.4	34.7
DPA	8.4	28.1
CLA	10.6	35.2
ETA	5.5	18.2
Adrenic	10.1	33.8
Oleic	4.9	16.3
EDA	11.7	39.1
Erucic	7.9	26.3

* Many of the LOD and LOQ values found in Table 1 may actually be lower than reported, due to standard degradation. Typical LOD values for charged aerosol detection are 1–10 ng on column.

Several oils and fats were processed and analyzed. In the initial oil hydrolyzation experiments, a solution of ethanol/water (3:2) was used. It was found that the oils did not hydrolyze well in this solution. With an exchange of isopropanol for the ethanol, it was identified that the isopropanol provided a greater yield of free fatty acids, and this solution was used for the hydrolyzation of the samples.

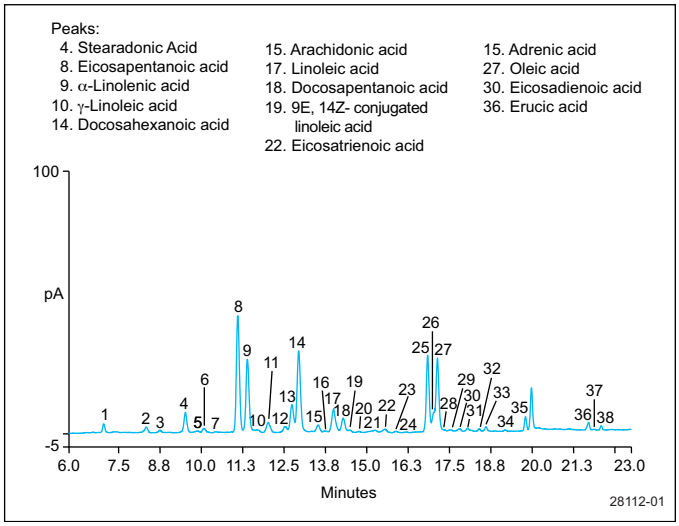
A chromatogram of hydrolyzed mustard oil is shown in Figure 5. This oil sample was found to contain a composition that is consistent with literature values[8,9] shown in brackets: 51% erucic [41–50%], 11% oleic [8–15%], 21% linoleic [13–20%], and 13% α-linolenic acids.

FIGURE 5. HPLC chromatogram of hydrolyzed mustard oil using a C30 150 × 4.5 mm, 5 µm column..



A hydrolyzed fish oil-based, commercially available supplement is shown in Figure 6. A large number of other free fatty acids may also be present with 24 unidentified peaks, in addition to the 14 evaluated in this study.

FIGURE 6. HPLC chromatogram of 20 µL hydrolyzed fish oil with addition of 200 µL isopropanol to aid in solubility. A total of 38 peaks were detected including all 14 standards.

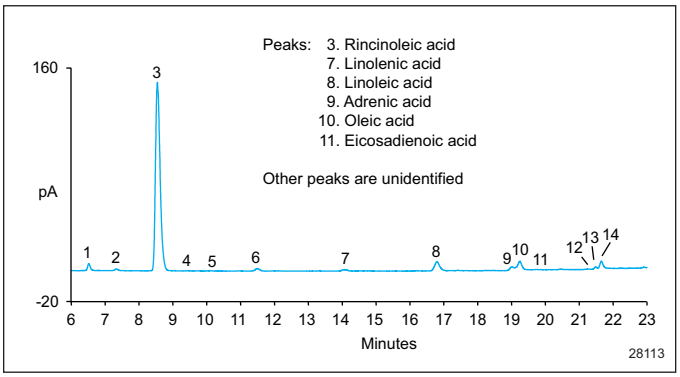


Twelve additional samples were hydrolyzed in a similar manner and the results are shown in Table 2 and presented based on the ratio of omega-3 to omega-6 ratio (highest to lowest). Fish oil had the highest ratio, explaining its use as an omega-3 oil supplement. Grass-fed beef was determined to have a ratio of approximately 1, close to literature values 0.3–0.7.[10] Interestingly, pasture-fed chicken was determined to have a ratio of approximately 0.62, significantly different than the commercial chicken omega fats ratio of approximately 0.05.[11]

Table 2. Percent Compositions of HPLC Analysis of Hydrolyzed Samples				
Sample	**Omega-3 (%)**	**Omega-6 (%)**	**Omega-9 (%)**	**3:6 Ratio**
Fish Oil	92.0	3.2	4.5	29.0
Fish, Flax, Borage Supplement	81.0	13.0	5.9	6.1
Flax Oil	59.0	22.0	19.0	2.7
Beef, Grass-fed	23.0	24.0	52.0	0.95
Avocado Oil	15.0	22.0	64.0	0.68
Chicken, Pastured	25.0	40.0	35.0	0.62
Mustard Oil	14.0	23.0	63.0	0.62
Canola Oil	13.2	33.2	53.6	0.39
Olive Oil	4.8	13.0	82.0	0.37
Walnut Oil	12.0	75.0	13.0	0.16
Castor Oil	4.2	62.0	34.0	0.067
Safflower Oil	0.71	17.0	82.0	0.041
Sesame Oil	1.5	61.0	38.0	0.024
Corn Oil	1.6	72.0	26.0	0.022

Castor oil was tested to evaluate the method's performance with other oils. A chromatogram for hydrolyzed castor oil is shown in Figure 7. Here, a single fatty acid, ricinoleate, was found to have 83 area percent (uncalibrated) of the fatty acids, which is similar to the reference value of 90%.[12] Ricinoleate, which contains a unique 12-hydroxy group, elutes earlier than the other free fatty acids quantified in this method.

FIGURE 7. HPLC chromatogram of hydrolyzed castor oil with ricinoleate-free fatty acid at 8.545 min.



Conclusions

Using this method, it is possible to obtain quantitative analyses of different omega-free fatty acids, including omega-3, -6, -7, and -9. Samples were hydrolyzed to separate the fatty acids from their glycerol backbone and analyzed directly using HPLC with charged aerosol detection. A wide variety of samples were analyzed, including animal- and plant-based oils, over-the-counter supplements, and meat fats. The mobile phase used here is compatible with mass spectrometry, which allows for the possibility of identifying unknown free fatty acids that may exist in a sample.

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