

Towards Standard-Free Quantitative and Qualitative Analysis in Liquid Chromatography

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INTRODUCTION

Natural plant oils are complex mixtures of various nonpolar compounds with triacylglycerols (TAGs) being the most significant components. They are an important source of essential fatty acids (FAs), e.g. linoleic and linolenic acid, as well as fat-soluble vitamins and other nonpolar compounds. Several diseases, i.e. coronary heart disease, dyslipidaemia, obesity, or inborn errors of metabolism can be caused by their imbalances in human diet.

Due to the chemical properties of TAGs, their analysis is highly challenging and their quantitation is complicated. Differences in chain lengths, in number and position(s) of the double bond(s), and other structural differences cause response factors (RFs) which differ widely between the individual compounds with common detectors such as UV, evaporative light scattering (ELSD) or atmospheric pressure chemical ionization mass spectrometry (APCI-MS). UV detection provides linear response, but due to the lack of strong chromophores, it offers very low sensitivity for both saturated and unsaturated TAGs, making their quantitation impossible. Detection by ELSD techniques is hampered by a nonuniform and nonlinear response. Furthermore, the RFs of individual FAs are very different and prevent a simple calculation of RFs of mixed-acid TAGs. APCI-MS provides a linear calibration and comparable sensitivity for saturated and unsaturated TAGs. RFs of mixed-acid TAGs can be calculated as the arithmetic mean of RFs of individual FAs.^{1,2} However, the RF in MS is critically dependent from molecular properties such as proton affinity, thus making quantitation based on reference standards mandatory.

Nevertheless, quantitation based on the relative peak areas while ignoring the different RFs is widely used in lipid analysis, even when quantifying without taking the RFs into account leads to inaccurate results. However, obtaining calibration curves for each single TAG is time consuming, expensive, or simply not possible because of the lack of TAG standards.

In contrast, charged aerosol detection combined with an inverse gradient provides universal and uniform response for nonvolatile compounds. The almost identical RF for all TAGs and other nonvolatile compounds offers the highly attractive opportunity of an easy quantitation without a large number of reference compounds based on the relative peak areas. Combined with the structural information provided by a high-resolution mass spectrometer, a system solution that delivers both qualitative and quantitative results within one run and without the need for extensive external calibration becomes feasible. How to approach this setup will be illustrated in the following by the analysis of FA triglycerols in edible oils of various flavors.

SYSTEM SETUP FOR CONVENTIONAL GRADIENT ELUTION AND COMPENSATION BY INVERSE GRADIENTS

The inverse gradient concept has been intensively tested and discussed together with the Thermo Scientific ESA Corona[®] *ultra*[™] CAD[®] Charged Aerosol Detector series. Just like an API mass spectrometer, this detector relies on mobile phase removal by a nebulizing process, leading to the formation of small particles from the residual analyte molecules. When colliding with a charged reactant gas, these particles become charged in the gas phase by nitrogen ions attached to the particle surface. These moving charges can be detected in an appropriate counter. As the signal intensity mostly only relies on the particle building efficiency, it strongly depends on the solvent composition. Compensating the mobile phase composition change during a gradient separation by merging the column effluent with the appropriate inverse amount of organic modifier will lead to constant nebulizing conditions, thus leading to a nearly uniform response. Figure 1 shows the solvent gradient compensation principle and the flow scheme for inverse gradients based on the UltiMate[®] 3000 ×2 Dual System with a Corona *ultra* CAD detector. The special design of the included DGP-3600RS pump integrates two ternary low-pressure gradient pumps in one housing. This allows for the easy delivery of the required compensation gradient using the second pump stream. In the conventional, noncompensated mode, only the right gradient pump is used. When compensating the analytical eluent composition with an inverse gradient, the left pump delivers the reverted solvent composition over a restrictor capillary. The difference in the gradient delay volume must be taken into account by an additional time shift in the compensation gradient program. In the compensation setup, analytical and compensation flow stream are merged by a T-piece prior to the Corona *ultra* CAD detector.

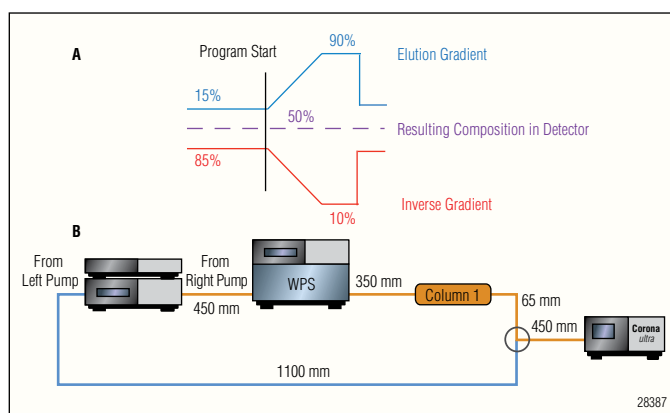


Figure 1. Inverse Gradient Concept (A) and instrumental configuration for a Corona *ultra* CAD detector (B).

As the Corona *ultra* CAD is a mass-sensitive detector and not concentration-dependent, diluting the sample by a factor of two by the merging flow behind the column will not affect detection sensitivity. Figure 2 illustrates this effect showing separation of six different diuretic compounds with detection using a Corona *ultra* CAD detector.

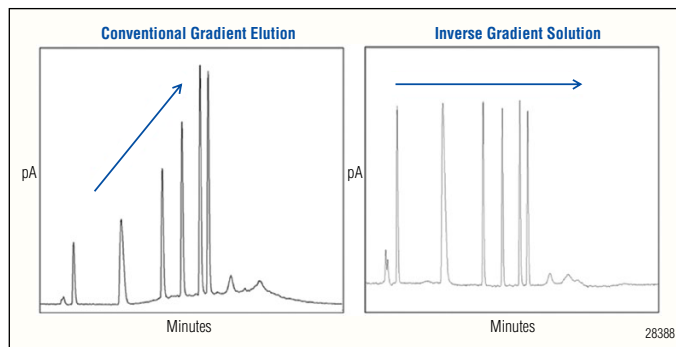


Figure 2. Illustration of the inverse gradient effect on the response of a mass-sensitive detector (Corona *ultra* CAD) for a separation of six diuretic compounds.

QUANTITATIVE ANALYSIS OF TAGS IN EDIBLE OILS USING CHARGED AEROSOL DETECTION

The first part of the feasibility study on standard-free quantitation addresses the applicability of the inverse-gradient concept to the separation of edible oils.

System

Thermo Scientific Dionex UltiMate® 3000 Dual Gradient Rapid Separation System consisting of the following modules: SRD-3600 Solvent Rack; DGP-3600RS Pump; WPS-3000TRS Wellplate Sampler; TCC-3000RS Thermostatted Column Compartment; Corona *ultra* CAD detector; Bruker micrOTOF-Q II with APCI source. All modules were connected with Thermo Scientific Dionex Viper™ Fittings of the Dionex UHPLC+ Solution "Inverse Gradient for Uniform Response" (typically 0.005 inch [0.13 mm] i.d).

LC/Charged Aerosol Detection Conditions

Columns: Thermo Scientific Dionex Acclaim® 120, 3 × 250 mm C18, 3 µm and Acclaim 120, 3 × 150 mm C18, 3 µm in series, connected with Viper fitting
 Eluent A: Acetonitrile (MS-grade)
 Eluent B: 2-Propanol (MS-grade)
 Flow: 1 mL/min (each)
 Gradient Program: cf. Table 1
 Max. Backpressure: 845 bar
 Injection Volume: 0.7 or 4 µL, resp.
 Column Temperature: 25 °C
 Data Collection Rate: 30 Hz
 Filter: Corona
 Standard Concentrations: 2–40 µg/µL in 2-propanol
 Oil Sample Concentrations: 40 µg/µL in 2-propanol

MS Conditions

APCI Ion Source Parameters

Nebulizer Pressure: 0.38 MPa
 Dry Gas: 3.0 L/min
 Dry Temperature: 300 °C
 Vaporizer Temperature: 400 °C
 Capillary Voltage: -4000 V
 End Plate Offset: -500 V
 Corona Current Setpoint: +4000 nA

Table 1. Gradient Elution and Separation Conditions of the Diuretics Sample Test Mix

Analytical Gradient Time [min]	% B	Compensation Gradient with Restrictor Capillary Time [min]	% B
-10	0	0	100
0	0	0	100
		2.15	100
106	69	108.15	31
109	0		
110	0	110	100

To achieve maximum peak capacity, a total column length of 40 cm was applied. The hydrophobic nature of the analytes made nonaqueous reversed-phase UHPLC separation necessary, with an elution gradient switching from acetonitrile to isopropanol (Table 1). Figure 3 illustrates efficient separation for three different edible oils (sesame, soybean, and sunflower)

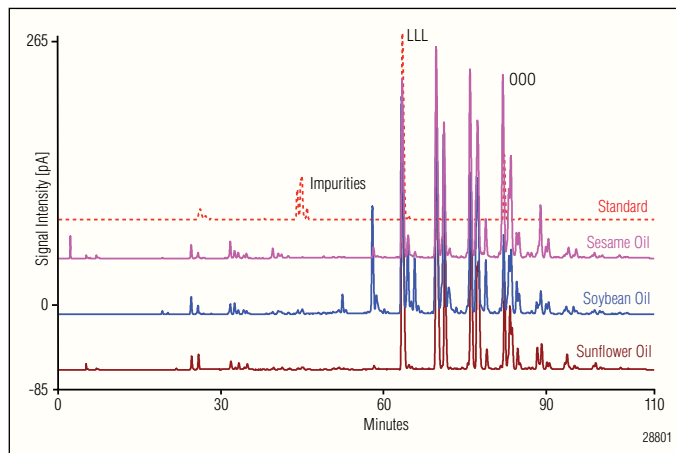


Figure 3. Separation of three different edible oils with charged aerosol detection and inverse gradient.

A comparison of the sunflower oil analysis with and without a compensating inverse gradient demonstrates the significant influence of the solvent composition on detector response. Figure 4 shows how the peak areas and signal heights across the elution window are balanced by the inverse gradient. As isopropanol content increases, peak response increases (red trace), while the signal level is equalized when the compensation gradient (green trace) is added.

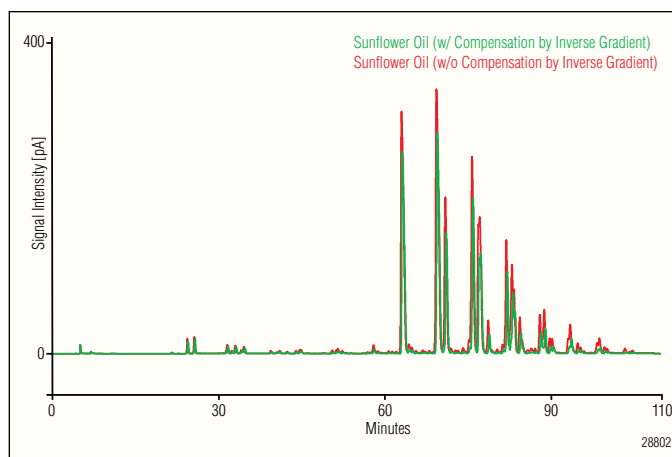


Figure 4. Comparison of detection with (green) and without (red) inverse gradient for solvent change compensation.

To prove the claim of uniform response for all relevant analytes, calibration curves of glycerol trilinoleate (LLL) and glycerol trioleate (OOO) have been determined and the RFs compared. In Figure 5, all calibration points for OOO and LLL are plotted into one diagram. The CAD detector provides a nonlinear signal response so that a polynomial fit function is required.

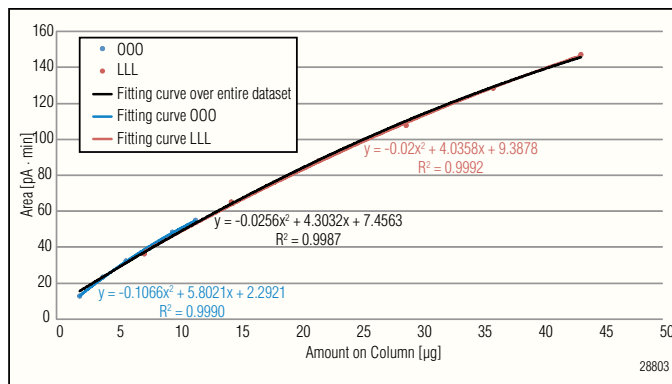


Figure 5. Calibration curve for glycerol trioleate (blue), glycerol trilinoleate (red), and over the entire dataset (black).

Table 2. Comparison of Calibration Results with Unified Calibration Curve

	Peak Area [pA-min]		Amount [µg] by 000 Calibration	Amount [µg] by LLL Calibration	Amount [µg] Using Unified Calibration Curve	
	000	LLL			000	LLL
Sunflower Oil	29.50	112.286	5.20	29.86	5.29	30.09
Soybean Oil	28.45	93.509	4.97	23.46	5.04	23.51
Sesame Oil	74.74	74.01	17.77	17.33	17.48	17.26

Table 3. Comparison of Relative Amounts with Relative Peak Areas

	Relative Amount [%] of 000 [µg 000/µg Sample] Using 000-Calibration	Relative Amount [%] of LLL [µg LLL/µg Sample] Using LLL-Calibration	Relative Amount [%] [µg Analyte/µg Sample] Using Unified Calibration Curve		Relative Peak Area [%]	
			000	LLL	000	LLL
Sunflower Oil	3.4	19.7	3.5	19.8	4.7	19.2
Soybean Oil	3.0	13.8	3.0	13.9	3.9	12.8
Sesame Oil	11.4	11.2	11.2	11.1	11.2	11.1

As shown in Table 2, the individual fitting curves for 000 (blue) and LLL (red) are so similar in terms of slope and correlation coefficient that one entire calibration curve over the total set of calibration points can be applied. This is emphasized by comparing the calibration results shown in Table 2, where the differences in the results between the individual and the unified calibration are within the limit of uncertainty for this method.

This outlines the uniform response behavior of the Corona *ultra* CAD detector when applying the inverse gradient concept. As a consequence, one calibration curve can be used for a semiquantitative determination of all compounds in these complex mixtures, including unknowns.

In Table 3 the correlation of relative amounts of TAGs with measured relative peak areas in the CAD detector is shown for the 3 different oils. It demonstrates adequate consistency of the data which is in agreement with published findings^{1,2}. Residual discrepancies between relative amount and relative peak area result from the calibration function characteristics of a CAD detector. The typical polynomial calibration function with nonzero intercept (Figure 5) is applied also for the calculation of relative TAG amounts. However, when relative peak areas are calculated simply the sum of all peak areas is considered as a base without taking into account the nonlinear signal response with intercept.

Nevertheless, a perfect match between simple peak area based relative quantitation and calibration based quantitation was found for 000 and LLL in sesame oil. As long as a certain inaccuracy in quantitation can be tolerated, it is thus even possible to quantify even unknown compounds when applying the Inverse Gradient for Uniform Response solution with the Corona *ultra* CAD detector. This is a significant step forward in achieving standard-free quantitative analysis in LC.

QUALITATIVE ANALYSIS OF TAGS IN EDIBLE OILS USING TIME-OF-FLIGHT MASS SPECTROMETRY

In classical chromatography, reference standards are not only used for correct amount determinations, but they are also essential for compound identification. Thus, an analytical solution free from reference standards must also have structural information to identify the analytes of interest. Here, high-resolving MS is a valuable tool for providing elemental composition by exact mass determination, and for structure elucidation by gas-phase fragmentation. Figure 6 shows the base peak current chromatogram of a UHPLC-APCI-TOF-MS experiment with soybean oil. For the sake of clarity, not all peaks have been annotated with the related FA glycerol triesters.

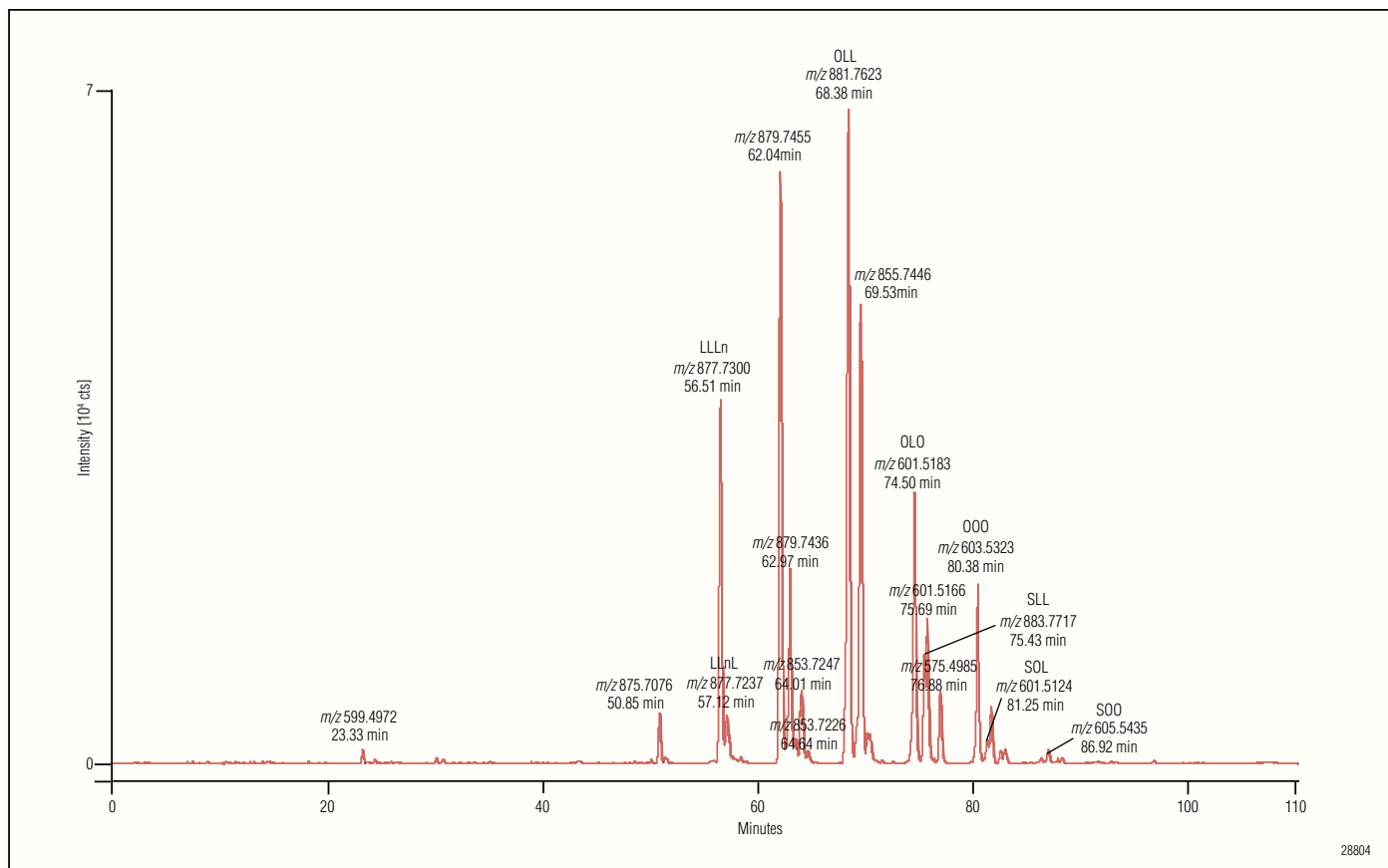


Figure 6. Analysis of soybean oil (see Figure 3) by UHPLC-APCI-TOF-MS.

The Figure 6 chromatogram illustrates the differences in the RFs of the individual FA glycerol esters, when being compared with the charged aerosol detection chromatogram (Figure 3, blue trace).

The combination of elemental composition by exact mass determination and fragmentation patterns of the glycerol esters enables the correct identification of analytical species even in most cases where isobaric compounds are detected. As Figure 7 illustrates, the peaks at the retention times 80.38 and 81.25 min have the same sum formula, as given by the exact mass of 885.783 m/z for the $[M+H]^+$ ion. However, the different fragmentations of the individual FA chains of the glycerol triesters allow an unambiguous differentiation of the glycerol trioleate (OOO) from the isobaric mixed glycerol ester from stearic, oleic, and linoleic acid (SOL).

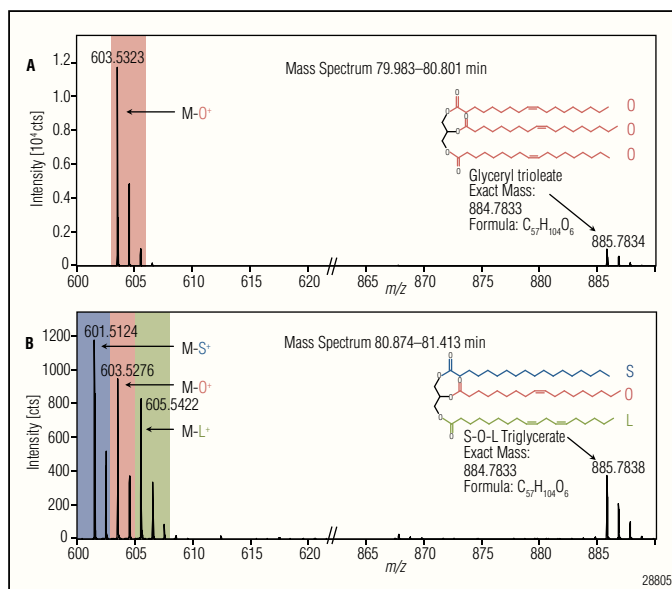


Figure 7. Structural information from in-source decay pattern of glycerol trioleate (A) and glycerol stearate/oleate/linoleate (B).

CONCLUSION

The charged aerosol detector is a valuable tool for developing a reference standard-free analytical solution. When applying the inverse gradient concept, this detector features almost uniform response for many analytes, which provides reliable quantitative results for unknown compounds. In combination with high-resolution MS which typically provides sufficient information for structure elucidation of unknown peaks, development of a time-effective analysis without the need for extensive external calibration is feasible.

REFERENCES

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2. Miroslav Lisa, Michal Holcapek, *J. Chromatography A*, 1198–1199, **2008**, 115–130.

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