

Sensitive HPLC Method for Triterpenoid Analysis Using Charged Aerosol Detection with Improved Resolution

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Overview

Purpose: To provide a sensitive analytical method for resolving and quantifying triterpenoids

Methods: A Thermo Scientific Acclaim C30 column provided high resolution of the different triterpenoids, and the Thermo Scientific Dionex Corona charged aerosol detector (CAD™) enabled the sensitive and direct quantitation of these analytes.

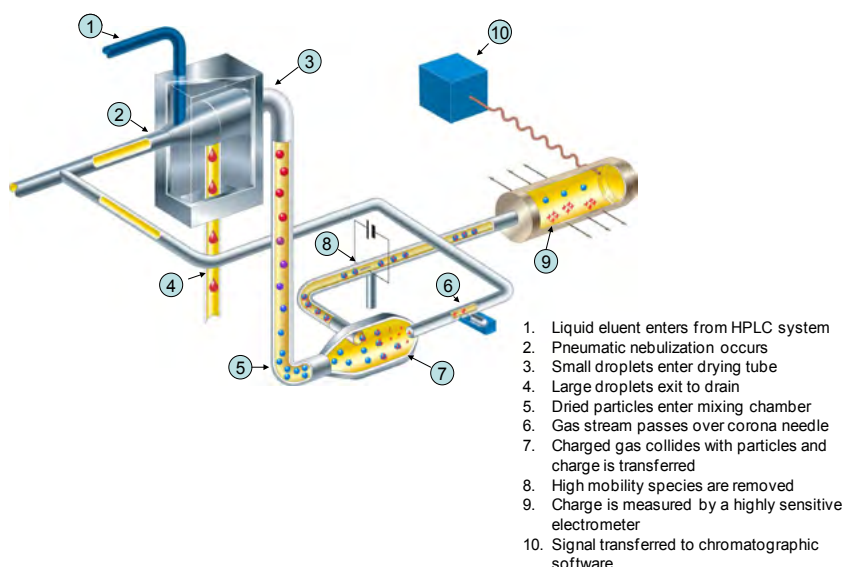
Results: Resolution between ursolic and oleanolic acid was greater than any reports in literature. No analyte derivatization was required, and limits of quantitation were < 2 ng on column. A sample of a dried spice and quantitation of ursolic acid in apple peel are provided.

Introduction

Triterpenoids are a class of naturally occurring compounds found in plants, and their beneficial effects on the immune system and in the treatment of cancer are currently being investigated. Some triterpenoids of interest include glycyrrhethinic, betulinic, oleanolic, and ursolic acids, where ursolic has been recently reported to exist at high concentration in apple skin.¹ Separation techniques using gas chromatography (GC), and micellar electrophoretic capillary chromatography, are either laborious (derivatization for GC) or have issues with reproducibility. With high performance liquid chromatography (HPLC) separation, a problem often encountered is poor resolution between oleanolic and ursolic acids, which tend to co-elute in many liquid chromatographic (LC) methods. One difficulty in making accurate determinations by UV is that these compounds do not have a strong chromophore moiety and sensitivity is compromised. A recent LC paper using a C18 column and UV absorption at 210 nm reported limits of detection of 50 and 135 ng on column for oleanolic and ursolic acids, respectively, with a resolution of 1.53 between these acids.² As reported in this work, a novel method using a C30 HPLC column and charged aerosol detection, with a resolution of 2.73 between oleanolic and ursolic acids is described. The limit of quantitation was also improved with an LOQ of 2 ng on column (o.c.) achieved for all four analytes. An example of triterpenoids in apple skin and basil is also demonstrated.

The charged aerosol detector is a sensitive, mass-based detector, especially well-suited for the determination of non-volatile and many semi-volatile analytes. As shown in Figure 1, the detector uses nebulization to create aerosol droplets. The mobile phase evaporates in the drying tube, leaving analyte particles, which become charged in the mixing chamber. The charge is then measured by a highly sensitive electrometer, providing reproducible, nanogram-level sensitivity. This technology has greater sensitivity and precision than evaporative light scattering (ELS), and it is simpler and less expensive to operate than a mass spectrometer (MS). Typical characteristics of chromatography with charged aerosol detection include: low-nanogram on column (o.c.) sensitivity, over four orders of magnitude of dynamic range, and high precision results, typically less than two percent of peak area RSD. Analyte response is also largely independent of chemical structure, providing clear relationships among different analytes in a sample

FIGURE 1: Schematic and functioning of charged aerosol detection.



Methods

Sample Preparation

Standards were dissolved in ethanol, except for ursolic acid, which was dissolved in acetone. Samples were sonicated in methanol/chloroform (1:1) for one hour, centrifuged at 13,000 g for 5 minutes, and diluted with one equivalent of methanol to assist in maintaining retention and analyte peak shape.

Liquid Chromatography

HPLC System: Thermo Scientific Dionex UltiMate 3000 SD
HPLC Column: Acclaim™ C30, 5 µm, 250 x 4.6 mm
Column Temperature: 30 °C
Mobile Phase A: 1 w/v% Ammonium acetate in water
Mobile Phase B: Acetonitrile/methanol (750 : 250)
Flow Rate: 1.0 mL/min
Detector: Corona™ ultra RS™
Nebulizer Temperature: 15 °C
Filter Setting: 4
Sample Temperature: Ambient
Injection Volume: 5.0 µL
Gradient:

Time (min)	%A	%B	Curve
0.0	92	8	--
20.0	20	80	1
20.0	92	8	5
25.0	92	8	5

Data Analysis

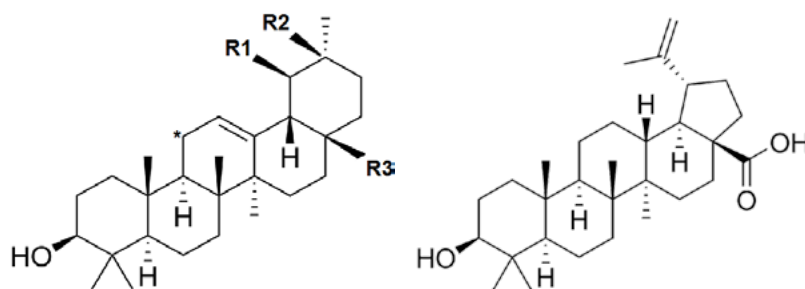
All HPLC chromatograms were obtained and compiled using Thermo Scientific Dionex Chromeleon 6.8 SR11 Chromatography Data Station.

Results

Method Development

The structures of the four triterpenoids are shown in Figure 2. These analytes have a very similar structure, often differing only by the position of a methyl group, making chromatographic separation difficult. One publication focused on the separation of oleanolic and ursolic acids, investigated nine different conditions, and at best achieved a resolution of 1.65 for these two analytes, respectively, using a C18 column.²

FIGURE 2: Chemical structures of the four triterpenoids included in this study.



Ursolic Acid, R1 = CH₃, R2 = H, R3 =COOH
Oleanolic Acid, R1 = H, R2 = CH₃, R3 =COOH
Glycyrrhetic Acid, R1=H, R2=COOH, R3=H, ketone at (*)

Betulinic Acid

A better separation for these compounds was achieved using a C30 column. The C30 solid phase is known to provide alternate selectivity over other column materials.³ Using the analytical conditions given above, this column gave a resolution of 2.73 for oleanolic and ursolic acids.

Standards were dissolved in ethanol (ursolic acid in acetone) to provide 4 to 2000 ng (o.c.) amounts. A chromatogram at 500 ng o.c. is shown in Figure 3, and calibration curves for the four triterpenoids (analyzed in triplicate) are presented in Figure 4. The peak area relative standard deviations for amounts greater than 30 ng o.c. was $\leq 5\%$. The data were fit to inverted second-order polynomials, yielding correlation coefficients, R^2 , greater than 0.998 for the four analytes.

FIGURE 3: Analysis of the four triterpenoids at 500 ng on-column.

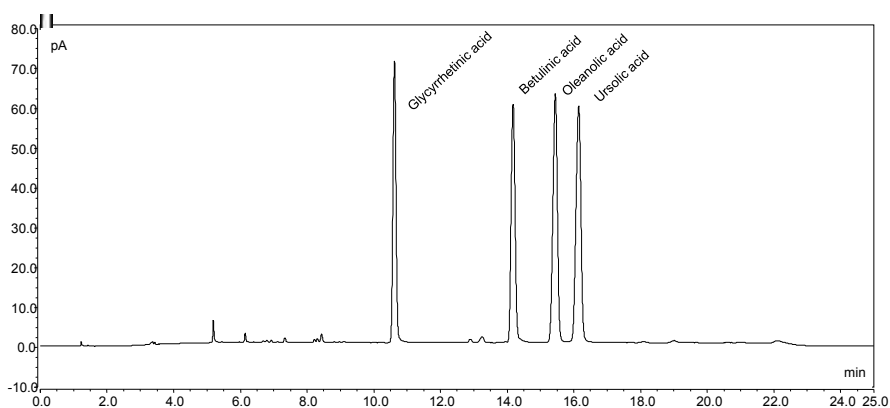
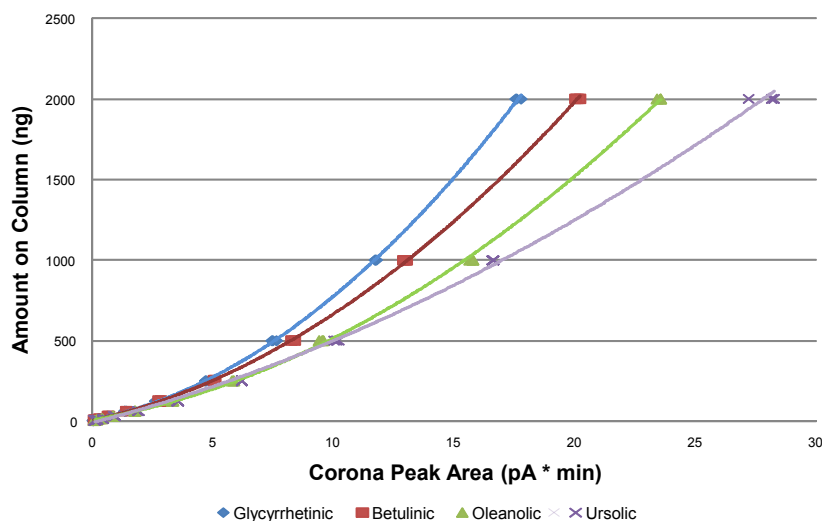


FIGURE 4: Calibration curves for glycyrrhetic, betulinic, oleanolic, and ursolic acids, from 4 to 2000 ng on-column.



Sensitivity values were determined using a signal-to-noise (S/N) of 3.3 for limit of detection (LOD) and 10.0 for limit of quantitation (LOQ). Using results from the 4 ng o.c. analysis, the LOQ was calculated to be < 2 ng o.c., and the LOD < 1 ng o.c., as shown in Table 2. Calibration precision (RSD) was $< 7\%$ for all analytes.

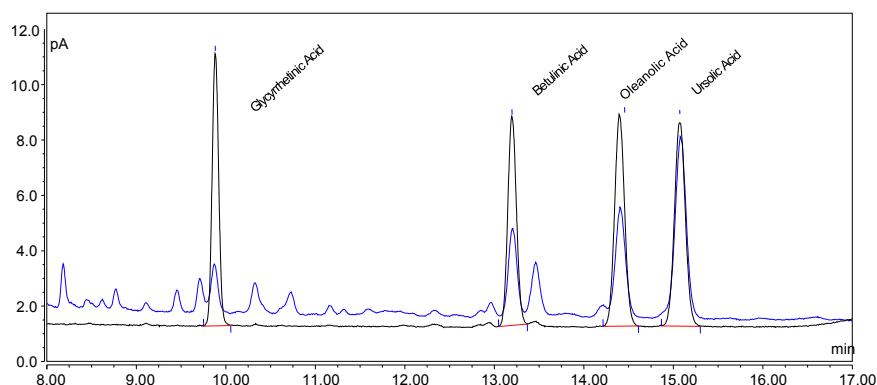
Table 2. Calibration precision and sensitivity values for triterpenoids by HPLC and charged aerosol detection.

Analyte	%RSD	LOD (ng o.c.)	LOQ (ng o.c.)
Glycyrrhetic acid	2.62	0.6	1.7
Betulinic acid	2.40	0.6	1.7
Oleanolic acid	4.82	0.5	1.5
Ursolic acid	6.82	0.5	1.5

Sample Analysis

Two samples were analyzed for triterpenoids: dried basil and apple peel. A qualitative sample preparation was performed on dried basil, using 19 mg/mL of basil in methanol/chloroform (1:1), diluted with one equivalent of methanol. A chromatogram, overlaid with a 32 ng o.c. standard chromatogram, is presented in Figure 5, and shows the presence of all four analytes, with ursolic and oleanolic acids being the two major triterpenoids, as reported by GC-FID.⁴

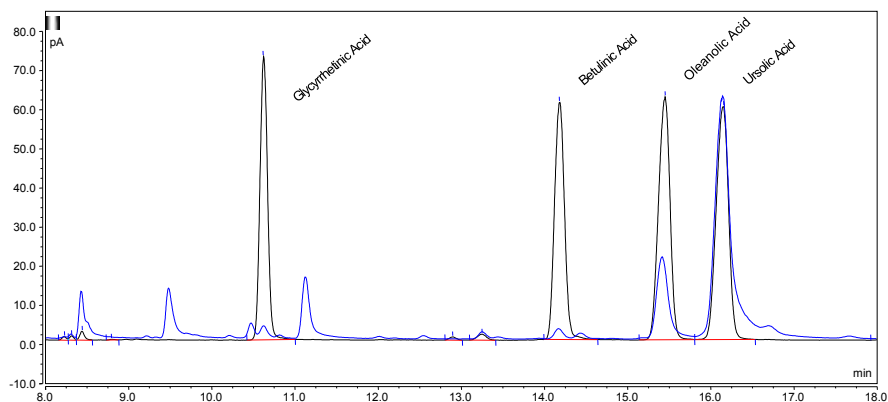
FIGURE 5. HPLC chromatogram of basil extract (blue) and 32 ng on-column triterpenoid standard solution (black).



The peel (3.037 g) from a red delicious apple was finely chopped and placed in a 30 mL centrifuge tube to which 12 mL of methanol/chloroform (1:1) was added. The mixture was sonicated for 1 hour, and then centrifuged at 13,000 g for five minutes. To a 500 μ L aliquot of supernatant, 500 μ L of methanol was added, mixed, and the resulting solution was analyzed.

A chromatogram of apple peel extract, overlaid with 500 ng o.c. standard, is shown in Figure 6. The four triterpenoids in the standard solution were found in the sample, along with a number of other peaks, which are unidentified. The amount of ursolic acid in the peel was found to be 0.14 (mass-percent) which is in good agreement to literature values (0.15%).⁵

Figure 6. HPLC chromatogram of apple peel extract (blue) and 500 ng on-column triterpenoid standard solution (black).



Method selectivity is the highest in the reported literature, with baseline separation of oleanolic and ursolic acids. Sensitivity is high, with an LOQ of <2 ng o.c., giving relative LOQ values of 0.0003 mass-percent. Method selectivity was not evaluated for other analytes within the ursane, oleanane, and lupane classes of triterpenes. Clearly, other compounds are present in the chromatography, and these can be easily identified with mass spectrometry, with which the chromatography is completely compatible. Corona charged aerosol can be used as the primary method of quantitation for the determination of these compounds, once identification has been established by an orthogonal approach, namely mass spectrometry.



Conclusion

A reversed-phase HPLC method was developed for the analysis of triterpenoids using the selectivity of a 3 μ m C30 column and the sensitivity and precision of the Corona ultra RS detector.

- The combination of acetonitrile and methanol with small-particle size C30 solid phase provides excellent separation of analytes.
- The method has unmatched resolution and detection limits for triterpenoids.
- Analysis is direct and samples do not require derivatization as is required for GC.

References

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