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A simplified HPLC-UV method for the analysis of triterpenoid acids from heritage apples (Malus domestica) from western North Carolina, USA

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ABSTRACT

Background: Pentacyclic triterpenoid acids are common in a number of food and spice plant species. Apples (*Malus domestica*) are the most common human food source for these potentially beneficial phytochemicals. Pre-20th century heritage apples have long been grown in mountainous western North Carolina and may be a wide-ranging source of these phytochemicals.

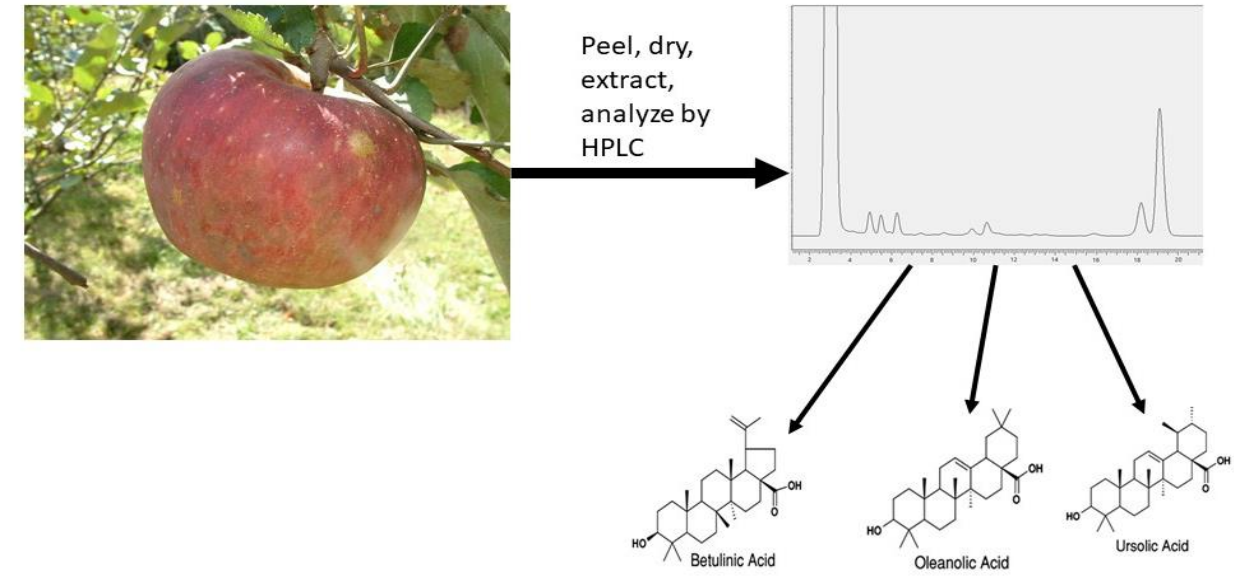
Objectives: Existing extraction and analytical methods were improved and used to assess the content of three triterpenoid acids in heritage apple cultivars grown in western North Carolina, USA.

Methods: Apples from a local farmers market were collected during the fall apple season. Apple peels were freeze dried, ground, and extracted with ethanol thrice. Extracts were analyzed by HPLC against external standards for betulinic, oleanolic, and ursolic acids.

Results: The improved method was used to extract and to analyze the triterpenoid acid levels in 16 heritage apple cultivars grown in the Appalachian region of western North Carolina. Total triterpenoid acids ranged from 2 to 29 mg/g dry weight of peels. Content did not vary by apple color or time of harvest. Russeted varieties contained noticeably less triterpenoids.

Conclusions: An improved and simplified method was used for the analysis of heritage apple varieties in western North Carolinas. A wide range of values was found for these compounds of increasing interest in the human diet and in human health.

Keywords: apples, phytochemicals, triterpenoids, ursolic acid, Appalachia



INTRODUCTION

Considerable scientific evidence shows that diets high in fruits and vegetables are associated with lower incidence of chronic conditions including cancer, cardiovascular disease, pulmonary dysfunction, and diabetes. [1-2] Research suggests that the high content of phytochemicals in fruits and vegetables are responsible for beneficial anti-inflammatory and antioxidant activities. [3]

Pentacyclic triterpenoid acids, a class of secondary metabolites formed by the cyclization of squalene, are widely distributed throughout the plant kingdom.[4-5] Due to their low polarity and lipophilic nature, they are principally found in surface cuticle waxes of leaves and

fruits. Waxes extracted from the cuticular layer of various plant tissues have been found to have a diversified chemical composition, consisting of hydrophobic waxes, aldehydes, ketones, alkanes, tocopherols, and triterpenoids. [6] Apple peels, rich in cuticular waxes, have been identified as promising sources of triterpenoids, particularly those belonging to the pentacyclic lupane, oleanane, or ursane series. [7, 8] These compounds possess numerous biological and pharmacological effects such as anti-inflammatory, immunoregulatory, antibacterial, antiviral, hypolipidemic, hypoglycemic and antitumor activities. [9-13] The major triterpenoid acids in apple peels are betulinic, oleanolic, and ursolic acids. See Figure 1.

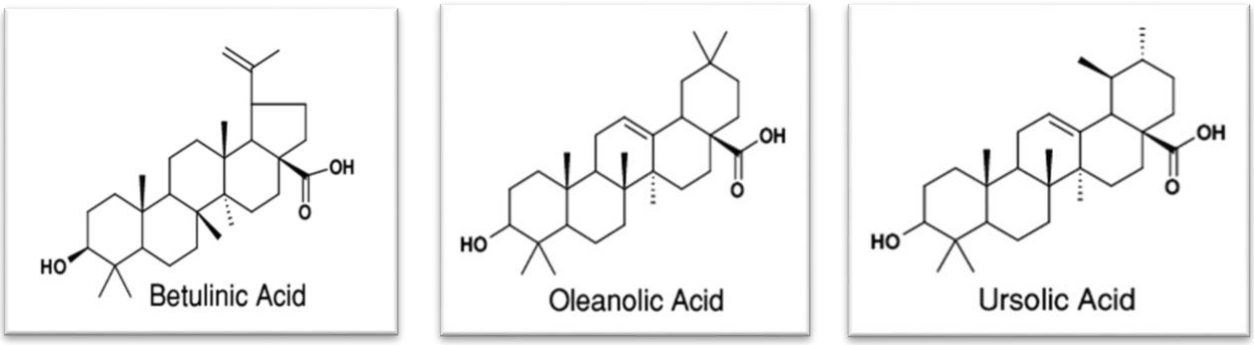


Fig 1. Chemical structure of betulinic acid, oleanolic acid and ursolic acid.

Triterpenoid acid concentrations can vary significantly between different apple cultivars; Belding et al. demonstrated that triterpenoid acid content could range from 32% of cuticular wax in Starkrimson cultivar to almost 70% in Pure Gold cultivar. [14] Andre et al found a 79-fold range of ursolic acid content across 109 varieties of apples. [15] North Carolina is the seventh largest producer of apples in the United States, with 70% of all apples in the state originating in Henderson County. [16] Early settlers in Appalachia frequently had small orchards on their properties with a range of varieties for their specialized purposes. It has been suggested that at one time many thousands of indigenous varieties were common in the area. [16, 17] Recently, a resurgent interest in the older pre-1900 heritage varieties have led to a number of orchardists curating and cultivating many of these varieties. An increased interest in craft hard ciders has supported this growth.

A range of analytical methods have been employed for the study of these compounds from apples. Extraction solvents have included ethyl acetate, [5, 18] hexane, [19] and ethanol. [8] Separation and analyses have been done by gradient HPLC procedures, [8, 18] NMR, [18] evaporative light scattering, [7], mass spectrometry [8, 20] and GC, TLC, and other methods. [4]

The aim of this research was to demonstrate a simplified and improved method for the extraction and quantitation of triterpenoid acids from apple peels and, specifically, to quantify betulinic, oleanolic, and ursolic acid content across North Carolina heritage apple cultivars using isocratic HPLC.

METHODS

Chemicals, Standards, and Reagents: HPLC grade methanol was obtained from OmniSolv, HPLC grade water from Ricca Chemical Company, ethanol (95%) from

BDH Chemical and acetic acid was from Amresco. Analytical standards used were betulinic acid (>98% purity) from AdooQ Bioscience, oleanolic acid and ursolic acid (>97% purity) were purchased from MP Biomedicals, LLC.

Collection and Sample Preparation: Apples were purchased at the Watauga County Farmers Market from Moretz Mountain Orchards in Boone, NC. Varietal names and heritage credentials were checked and in a few cases slightly renamed to agree with C. Lee Calhoun, a recognized authority in southern apples. [17] Author R.J. independently assessed and verified the varietal identities. Apples were hand peeled using a standard vegetable peeler (Cuisinart). All peels were stored at -80°C. For analysis, peel samples were freeze-dried for 72 hrs using a Labconco FreeZone® Freeze Dry System.

Separation and Extraction: The extraction method is a modification of the method of McGhie et al. [8] Freeze dried peel samples were blended using a food processor (Kitchenaid) until evenly pulverized to nearly a powder-like fineness and 5.00 g was then blended with 100 mL of ethanol using a Waring 7010G blender with Waring MC3 metal container. Aluminum foil was used under the neoprene blender lid to prevent contamination of the extract by the neoprene. The mixtures were left to soak for 24 hrs at 4° C, then filtered under vacuum. The residue was blended twice more with 100 mL ethanol and filtered. The combined ethanol extract was dried by rotary evaporation (Buchi Rotavapor RII) at 50° C, which was then resuspended in methanol (20 mL) and stored at -20° C prior to analysis.

HPLC Analysis: Prior to analysis by HPLC, methanolic extracts were diluted 1:10 in methanol and filtered through a 25 mm syringe filter with 0.45 µm polypropylene membrane (VWR international, USA). Aliquots of diluted extract were analyzed using an HPLC system from Waters Company (Milford, MA) fitted with a C18 column (Waters XBridge™ C18, 3.5 µm, 4.6 x 150 mm). Injections of 20 µL were made using a Waters 2707 autosampler, with a flow rate of 0.5 mL/min^{-1}. The column temperature was set at 30°C with a detection wavelength of 207 nm (Waters 2489 UV/visible detector). The isocratic mobile phase used was 85% methanol, 15% water, and 0.1% acetic acid. Triterpenoid acid content was quantified using ursolic, betulinic, and oleanolic acid external standards. This method is a simplification of the gradient method of Jäger. [5]

RESULTS

Optimization of the Chromatographic Conditions: HPLC parameters were optimized by the selection of mobile phase, column temperature, and detection wavelength. The final mixture of methanol and water (85:15) maximized the separation of oleanolic and ursolic acids, with a small amount of acetic acid (0.1% v/v) to sharpen peak shapes and improve resolution. The detection wavelength of 207 nm was selected as a compromise between the maximum adsorption wavelength of the triterpenoid acids and the opaqueness of methanol at these extremely low wavelengths. The chromatographic results are shown in Figures 2 for standards and in Figure 3 for an apple peel sample.

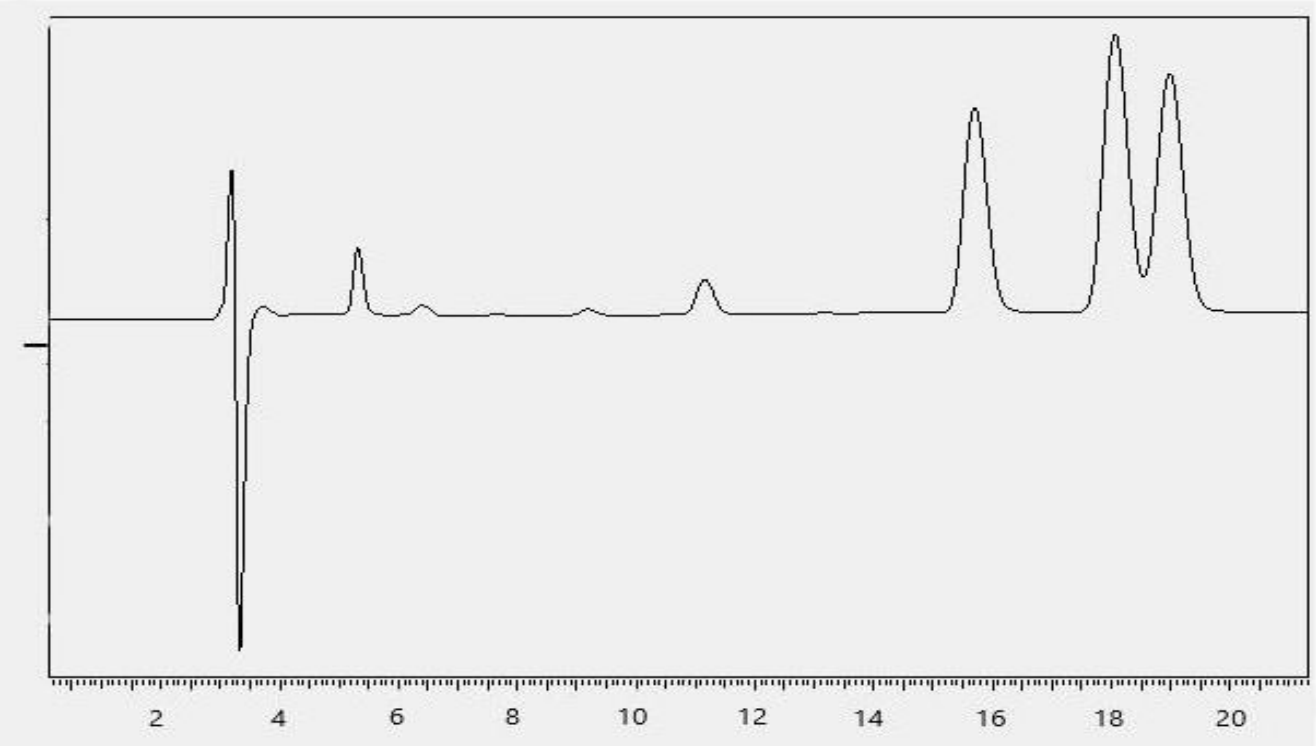


Fig 2. Chromatogram of Standards. Retention times: Betulinic (15.7), oleanolic (18.1), and ursolic (19.0)

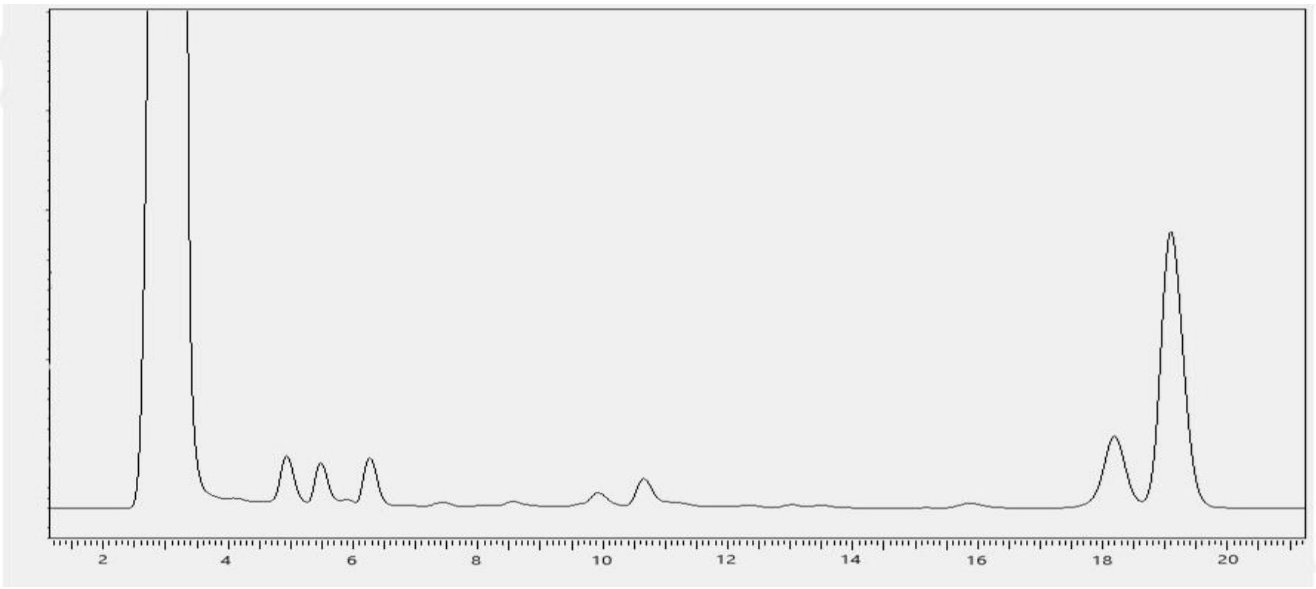


Fig 3. Chromatogram of Virginia Gold peel sample. Retention times: Betulinic (15.9), oleanolic (18.2), and ursolic (19.1)

Assessment of Triterpenoid Contents in Apple Peel Extracts: The developed analytical method was applied for the determination of the three triterpenoid acids in the 16 heritage cultivars collected. All three compounds were detected from every sample. See Table 1. Total triterpenoid content ranged from 2 mg/g dried weight (Roxbury Russet) to 29 mg/g dried weight (Granny Smith). Betulinic, oleanolic, and ursolic acid averaged 0.2, 3.3, and 14.7 mg/g dried weight, respectively across all 16 varieties. A selection of apples developed or discovered in the 20th century were also evaluated. See Table 2.

Table 1. Pre-20th Century Heritage Apple Peel Triterpenoid Acid Content (mg/g dry weight) from western North Carolina

Apple Variety	Color	Harvest Date	Betulinic acid	Oleanolic acid	Ursolic acid	Total triterpenoids
Granny Smith	green	September to October	0.31	5.89	22.97	29.17
Rome Beauty	dark red	September to October	0.26	4.49	19.94	24.69
King Luscious	dull red over yellow green	October	0.21	5.01	19.05	24.27
Wolf River	greenish with stripes of red	late September	0.18	4.46	19.37	24.01
Red Delicious	red	late September	0.25	4.43	18.16	22.84
Buckingham	yellow with red striping	early fall	0.25	4.05	16.80	21.10
Fameuse	alternating red and green	early October	0.24	1.61	17.52	19.37
Magnum Bonum	red	September to October	0.25	3.78	14.42	18.45
Virginia Beauty	alternating red and green	October-February	0.33	3.62	13.82	17.77
Dula Beauty	red	late fall to early winter	0.16	4.23	13.21	17.60
Stayman	greenish with stripes of red	October	0.21	2.06	14.62	16.89
McIntosh	alternating red and green	September to December	0.22	2.23	13.77	16.22
York Imperial	yellow with stripes -red	November – December	0.14	2.84	12.50	15.48
Arkansas Black	dark red	October	0.22	1.71	12.65	14.58
Royal Limbertwig	greenish yellow with red blush	October to November.	0.22	1.72	5.55	7.49
Roxbury Russet	greenish to gold	September to October	0.23	0.65	1.12	2.01

Table 2. 20th Century Variety Apple Peel Triterpenoid Acid Content (mg/g dry weight) from western North Carolina

Variety	Betulinic acid	Oleanolic acid	Ursolic acid	Total Triterpenoids
Virginia Gold	0.67	7.09	28.51	36.27
Sweet Sixteen	0.35	6.61	26.42	33.38
Shizuka	0.39	5.56	18.12	24.07
Jonagold	0.39	4.18	18.38	22.95
Liberty	0.24	4.86	17.15	22.25
Cameo	0.28	4.81	15.40	20.49
GoldRush	0.27	4.25	15.31	19.83
Jumbo	0.21	4.00	14.77	18.98
Jonamac	0.26	2.21	16.04	18.52
Honeycrisp	0.19	2.96	14.90	18.05
Red Gold	0.26	2.13	14.03	16.42
Geneva Early	0.21	3.46	12.65	16.32

Blushing Golden	0.17	3.64	12.49	16.30
Mutsu	0.23	3.55	11.99	15.78
Criterion	0.26	3.70	11.28	15.24
Cortland	0.26	3.22	11.37	14.85
Empire	0.17	1.61	12.83	14.61
Enterprise	0.18	2.62	10.90	13.70
Jonared	0.27	3.11	10.19	13.57
Gala	0.15	1.46	9.69	11.30

DISCUSSION

The method developed, based on previous methods of extraction and analysis appears to be stable and reliable. While the method is not especially sensitive, it is appropriate for the analysis of fruit samples. Earlier attempts were made to use acetonitrile as the HPLC solvent. This more transparent solvent at the lower UV wavelengths would have increased sensitivity; however, adequate separation was not achievable with this solvent and methanol was substituted successfully.

Previous studies have identified triterpenoid compounds in apple cuticles. [8, 15, 20-21] This study examined triterpenoid acids in 16 heritage cultivars from western North Carolina. Previous methods employed solvents such as chloroform and hexane to dissolve the apple peel wax layer, whereas in this study, peels were extracted by repeated extractions with less toxic ethanol, a modified method adapted from McGhie et al. [8]

Yamaguchi et al. reported an ursolic acid content of 0.7% of dry peel weight in Fuji, [19] and Jager et al. reported an ursolic acid range of 0.2 – 2.1% and an average oleanolic value of 0.28%. [5] These ursolic and oleanolic acid values are comparable to the ursolic acid average of 1.42% of dry peel weight and oleanolic acid average of 0.32% from this study. In common with Jager et al, a wide range in ursolic and oleanolic acid values is reported in this study, from 0.11% - 2.3% and 0.07 - 0.59%, respectively. Butkevičiūtė et al reported average total triterpenoid values of about 0.7% of dry weight, about half of the average values of this study. [21] Betulinic acid values were an order of magnitude lower than oleanolic acid values.

Russeted cultivars such as Roxbury Russet and Sour Rusty Coat had less triterpenoid acids compared to non-russeted cultivars such as Virginia Gold and Granny Smith. This may be due to a lesser amount of cuticular waxes present in russeted cultivars. No particular pattern of triterpenoids was associated with the color of the apple or between early and late season varieties. Dashbaldan et al recently showed that the level of triterpenoids approximately doubles over the four months of apple maturation. [22] Butkevičiūtė found that triterpenoid content decays during controlled atmosphere storage for eight months. [23] Further work to assess the quantity of triterpenoid acids as a fraction of the cuticular wax across varieties and over time would certainly be warranted.

Western North Carolina is in the Appalachian Mountains and hence is cooler than comparable latitudes of the eastern United States and is more conducive to apple growing. Farmers markets in the area have seen a resurgence of interest in older heritage apple varieties coupled with an increased interest in craft heritage hard ciders. It is hoped that the information presented here adds to the appeal of the apples from this region. A simplified method such as this will improve the survey of these important compounds in the wide range of heritage apples and identify those varieties with particularly high levels. As newer apple varieties are being developed, trademarked, and marketed across the

country, the levels of these important phytochemicals may become of more interest to those in breeding programs. This method may also be easily modified for the analysis of apple pomace and for related crops such as pears.

CONCLUSIONS

In this study, a simple extraction and HPLC method has been developed for the determination of triterpenoid acids in apple peels. Peels from North Carolina heritage apple varieties are a wide-ranging source of triterpenoid acids.

List of Abbreviations: GC, gas chromatography; HPLC, high pressure liquid chromatography; UV, ultraviolet.

Author Contributions: Weston Petroski, Kimberly Bechtold, Leslie McCullen, Samantha Lambiase, and Brooke Wilson performed the research. Ronald Joyner reviewed the apple nomenclature and variety names. Martin Root supervised the research and wrote the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

REFERENCES

1. Gan Y, Tong X, Li L, Cao S, Yin X, Gao C, Herath C, Li W, Jin Z, Chen Y, Lu Z: Consumption of fruit and vegetable and risk of coronary heart disease: A meta-analysis of prospective cohort studies. Int J Cardiol 2015,183:129-137. DOI: https://doi.org/10.1016/j.ijcard.2015.01.077
2. Aune D, Giovannucci E, Boffetta P, Fadnes LT, Keum N, Norat T, Greenwood DC, Riboli E, Vatten LJ, Tonstad S: Fruit and vegetable intake and the risk of cardiovascular disease, total cancer and all-cause mortality-a systematic review and dose-response meta-analysis of prospective studies. Int J Epidemiol 2017,46(3):1029-1056. DOI: https://doi.org/10.1093/ije/dyw319
3. Phan MAT, Paterson J, Bucknall M, Arcot J: Interactions between phytochemicals from fruits and vegetables: Effects on bioactivities and bioavailability. Crit Rev Food Sci Nutr 2018,58(8):1310-1329. DOI: https://doi.org/10.1080/10408398.2016.1254595
4. Xu C, Wang B, Pu Y, Tao J, Zhang T: Techniques for the analysis of pentacyclic triterpenoids in medicinal plants. J Sep Sci 2018,41(1):6-19. DOI: https://doi.org/https://doi.org/10.1002/jssc.201700201
5. Jager S, Trojan H, Kopp T, Laszczyk MN, Scheffler A: Pentacyclic triterpene distribution in various plants - rich sources for a new group of multi-potent plant extracts. Molecules 2009,14(6):2016-2031. DOI: https://doi.org/10.3390/molecules14062016
6. Liu J: Oleanolic acid and ursolic acid: Research perspectives. J Ethnopharmacol 2005,100(1-2):92-94. DOI: https://doi.org/10.1016/j.jep.2005.05.024
7. Lesellier E, Destandau E, Grigoras C, Fougère L, Elfakir C: Fast separation of triterpenoids by supercritical fluid chromatography/evaporative light scattering detector. J Chrom A 2012,1268:157-165. DOI: https://doi.org/https://doi.org/10.1016/j.chroma.2012.09.102
8. McGhie TK, Hudault S, Lunken RC, Christeller JT: Apple peels, from seven cultivars, have lipase-inhibitory activity and contain numerous ursenoic acids as identified by LC-ESI-QTOF-HRMS. J Agric Food Chem 2012,60(1):482-491. DOI: https://doi.org/10.1021/jf203970j
9. Jang S-E, Jeong J-J, Hyam SR, Han MJ, Kim D-H: Ursolic acid isolated from the seed of *cornus officinalis* ameliorates colitis in mice by inhibiting the binding of lipopolysaccharide to toll-like receptor 4 on macrophages. J Agr Food Chem 2014,62(40):9711-9721. DOI: https://doi.org/10.1021/jf501487v
10. Yin R, Li T, Tian JX, Xi P, Liu RH: Ursolic acid, a potential anticancer compound for breast cancer therapy. Crit Rev Food Sci Nutr 2018,58(4):568-574. DOI: https://doi.org/10.1080/10408398.2016.1203755

11. Nguyen HN, Ullevig SL, Short JD, Wang L, Ahn YJ, Asmis R: Ursolic acid and related analogues: Triterpenoids with broad health benefits. Antioxidants 2021,10(8):1161. DOI: https://doi.org/10.3390/antiox10081161
12. Kunkel SD, Elmore CJ, Bongers KS, Ebert SM, Fox DK, Dyle MC, Bullard SA, Adams CM: Ursolic acid increases skeletal muscle and brown fat and decreases diet-induced obesity, glucose intolerance and fatty liver disease. PLoS One 2012,7(6):e39332. DOI: https://doi.org/10.1371/journal.pone.0039332
13. Silva FS, Oliveira PJ, Duarte MF: Oleanolic, ursolic, and betulinic acids as food supplements or pharmaceutical agents for type 2 diabetes: Promise or illusion? J Agric Food Chem 2016,64(15):2991-3008. DOI: https://doi.org/10.1021/acs.jafc.5b06021
14. Belding RD, Blankenship SM, Young E, Leidy RB: Composition and variability of epicuticular waxes in apple cultivars. J Am Soc Hort Sci 1998,123(3):348-356. DOI: https://doi.org/10.21273/JASHS.123.3.348
15. Andre CM, Greenwood JM, Walker EG, Rassam M, Sullivan M, Evers D, Perry NB, Laing WA: Anti-inflammatory procyanidins and triterpenes in 109 apple varieties. J Agric Food Chem 2012,60(42):10546-10554. DOI: https://doi.org/10.1021/jf302809k
16. North Carolina Department of Agricultural and Consumer Services: Goodness grows in North Carolina: Apple Outline. [http://www.ncagr.gov/agscool/teacher/commodities/apple.htm]. Retrieved March 5, 2022.
17. Calhoun CL, Old Southern Apples. 2nd ed.; White River Junction, Vermont: Chelsea Green Publishing Company; 2010.
18. He X, Liu RH: Triterpenoids isolated from apple peels have potent antiproliferative activity and may be partially responsible for apple's anticancer activity. J Agric Food Chem 2007,55(11):4366-70. DOI: https://doi.org/10.1021/jf063563o
19. Yamaguchi H, Noshita T, Kidachi Y, Umetsu H, Hayashi M, Komiyama K, Funayama S, Ryoyama K: Isolation of ursolic acid from apple peels and its specific efficacy as a potent antitumor agent. J Health Sci 2008,54(6):654-660. DOI: https://doi.org/10.1248/jhs.54.654
20. Oszmiański J, Lachowicz S, Gławdel E, Cebulak T, Ochmian I: Determination of phytochemical composition and antioxidant capacity of 22 old apple cultivars grown in Poland. Eur Food Res and Tech 2018,244(4):647-662. DOI: https://doi.org/10.1007/s00217-017-2989-9
21. Butkevičiūtė A, Liaudanskas M, Kviklys D, Zymonė K, Raudonis R, Viškelis J, Uselis N, Janulis V: Detection and analysis of triterpenic compounds in apple extracts. Inter J Food Properties 2018,21(1):1716-1727. DOI:
22. https://doi.org/10.1080/10942912.2018.1506478
23. Dashbaldan S, Pączkowski C, Szakiel A: Variations in triterpenoid deposition in cuticular waxes during development and maturation of selected fruits of rosaceae family. Int J Mol Sci 2020,21(24):9762. DOI: https://doi.org/10.3390/ijms21249762
24. Butkeviciute A, Viskelis J, Liaudanskas M, Viskelis P, Bobinas C, Janulis V: Variation of triterpenes in apples stored in a controlled atmosphere. Molecules 2021,26(12):3639. DOI: https://doi.org/10.3390/molecules26123639