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# Profiling LC-DAD-ESI-TOF MS Method for the Determination of Phenolic Metabolites from Avocado (*Persea americana*)

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**ABSTRACT:** A powerful HPLC-DAD-ESI-TOF MS method was established for the efficient identification of the chemical constituents in the methanolic extracts of avocado (*Persea americana*). Separation and detection conditions were optimized by using a standard mix containing 39 compounds belonging to phenolic acids and different categories of flavonoids, analytes that could be potentially present in the avocado extracts. Optimum LC separation was achieved on a Zorbax Eclipse Plus C18 analytical column (4.6 × 150 mm, 1.8 μm particle size) by gradient elution with water + acetic acid (0.5%) and acetonitrile as mobile phases, at a flow rate of 1.6 mL/min. The detection was carried out by ultraviolet−visible absorption and ESI-TOF MS. The developed method was applied to the study of 3 different varieties of avocado, and 17 compounds were unequivocally identified with standards. Moreover, around 25 analytes were tentatively identified by taking into account the accuracy and isotopic information provided by TOF MS.

**KEYWORDS:** Liquid chromatography, time-of-flight-mass spectrometry, phenolic compounds, avocado, *Persea americana*, secondary metabolites

## INTRODUCTION

Phenolic compounds are a wide group of secondary metabolites that can be found in plants, such as simple phenols, phenolic acids, flavonoids, coumarins, tannins, stilbenes, lignans, lignins.[1] These compounds can perform several functions in plants, such as protective agents against UV light, taking part in fertilization by attracting pollinators, and being components of pigments, essences, flavors, etc. In foods and beverages, phenolic compounds may contribute to oxidative stability and organoleptic characteristics (bitterness, flavor, astringency, etc.).[2−4] Numerous investigations have shown that, in humans, phenols exhibit potentially positive effects such as antimicrobial,[5] cardioprotective, antiallergenic, and anti-inflamatories activities,[6,7] among others. These beneficial effects of phenols have been traditionally related to their strong antioxidant activity, that is, their ability to scavenge oxygen radicals and other reactive species.[8,9]

An important source of phenolic compounds are the fruits and vegetables produced in the Mediterranean basin. In the current study, we focused on avocado fruit (*Persea americana*) because it is an important crop of the tropical coast of Granada (Spain) and it is increasingly valued by consumers, not only for its unique flavor and texture but also for its reported health benefits.[10−12] Avocado is an evergreen tree[13] native to Colombia, Mexico, and Venezuela. There are many varieties (e.g., Fuerte, Hass, Pinkerton, Bacon, Azteca, Ettinger, or Rincon), and all of them show common characteristics: oval form, pale green pulp, one large seed only, rough or smooth surface, etc.

The qualitative and quantitative determination of these phenolic compounds in fruits and vegetables is therefore very important, and several methods have been already described in literature.[1,2,14,15] Traditionally, spectrophotometric methods and thin layer chromatography (TLC) were used, but later, the need to separate and identify phenols individually caused the replacement of traditionally methods by other techniques, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) coupled to different detectors. Furthermore, capillary electrophoresis (CE) has been recently applied to the analysis of phenolic compounds from fruits and vegetables.[16]

The phenolic fraction from avocado has not been studied in depth so far; there are several very interesting papers wherein the reader can find some information about the composition of avocado concerning phenolic compounds,[11,17−21] but there is not a detailed description about the pseudopolar fraction of this important fruit. In some of the mentioned papers, the authors studied the phenolic composition of different fruits, vegetables, or plant food material in general, and they determined just a few compounds in avocado samples. Torres et al.[17] were pioneers describing the phenolic acids that can be found in avocado fruit. Ding et al.[11], however, in a very complete study, established a possible connection between some phytochemicals present in avocado and its chemopreventive characteristics.

The aim of this work was to develop a powerful HPLC-DAD-ESI-TOF MS method for the characterization of phenolic compounds from avocado fruit. The potential of this separation technique coupled to DAD and TOF detectors was evaluated by using a standard mix containing 39 compounds belonging to different families of phenols. After optimization of the separation and detection conditions, the optimum method was applied to the analysis of three varieties of avocado fruit (Hass, Lamb−Hass, and Rugoro), achieving the identification of an important number of compounds in the analyzed extracts.

## MATERIALS AND METHODS

**Chemicals and Standards.** The solvents used for the sample extraction were methanol and ethanol, which were purchased from Panreac (Barcelona, Spain); and acetone and ethyl acetate, which were

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**Table 1. MS Signal Observed for the 39 Compounds Included in the Standard Mixture in Negative and Positive Ion Polarity**[a]

| | Compound | Formula | Selected ion | m/z experimental | m/z theoretical | Error (ppm) | mSigma Value | Retention time (min) | Fragments in source |
|---|---|---|---|---|---|---|---|---|---|
| ♣ | Gallic acid | $C_7H_6O_5$ | $[M-H]^-$ | 169.0147 | 169.0142 | -2.6 | 9.5 | 3.4 | 125.0270 |
| | | | $[M+H]^+$ | 171.0286 | 171.0288 | 1.1 | 5.1 | | - |
| ♣ | Protocatechuic acid | $C_7H_6O_4$ | $[M-H]^-$ | 153.0191 | 153.0193 | 1.8 | 7.7 | 5.2 | 109.0262 |
| | | | $[M+H]^+$ | 155.0337 | 155.0339 | 1.3 | 7.1 | | 137.0238 |
| ♣ | Gentisic acid | $C_7H_6O_4$ | $[M-H]^-$ | 153.0187 | 153.0193 | 4.5 | 7.2 | 6.4 | 109.0259 |
| | | | $[M+H]^+$ | 155.0341 | 155.0339 | -1.3 | 4.1 | | - |
| ♣ | 4-Hydroxybenzoic acid | $C_7H_6O_3$ | $[M-H]^-$ | 137.0245 | 137.0244 | -0.7 | 5.6 | 6.9 | 93.0274 |
| | | | $[M+H]^+$ | 139.0386 | 139.0389 | 2.1 | 6.1 | | 121.0305 |
| ♣ | Chlorogenic acid | $C_{16}H_{18}O_9$ | $[M-H]^-$ | 353.0886 | 353.0878 | -2.4 | 7.1 | 7.5 | 191.0560 |
| | | | $[M+H]^+$ | 355.1021 | 355.1024 | 0.8 | 24.9 | | 163.0380<br>218.9902<br>291.0845 |
| ▲ | Catechin | $C_{15}H_{14}O_6$ | $[M-H]^-$ | 289.0712 | 289.0718 | 2.2 | 10.2 | 7.6 | 245.0818 |
| | | | $[M+H]^+$ | 291.0863 | 291.0863 | 0.0 | 11.0 | | 123.0447<br>139.0387<br>163.0385 |
| ♣ | Vanillic acid | $C_8H_8O_4$ | $[M-H]^-$ | 167.0347 | 167.0350 | 1.6 | 4.8 | 8.1 | - |
| | | | $[M+H]^+$ | 169.0498 | 169.0495 | -1.8 | 5.1 | | 151.0385 |
| ♣ | Caffeic acid | $C_9H_8O_4$ | $[M-H]^-$ | 179.0355 | 179.0350 | -2.9 | 9.3 | 8.3 | 135.0440 |
| | | | $[M+H]^+$ | 181.0493 | 181.0495 | 1.5 | 33.4 | | 135.0445<br>163.0384 |
| ♣ | Syringic acid | $C_9H_{10}O_5$ | $[M-H]^-$ | 197.0447 | 197.0455 | 4.2 | 4.2 | 8.9 | - |
| | | | $[M+H]^+$ | 199.0611 | 199.0601 | -5.0 | 7.1 | | - |
| ♣ | Homovanillic acid | $C_9H_{10}O_4$ | $[M-H]^-$ | 181.0504 | 181.0506 | 1.0 | 8.9 | 8.9 | 137.0618<br>122.0372 |
| | | | $[M+H]^+$ | 183.0654 | 183.0652 | -1.1 | 5.9 | | 137.0619 |
| ▲ | Epicatechin | $C_{15}H_{14}O_6$ | $[M-H]^-$ | 289.0715 | 289.0718 | 0.9 | 1.9 | 9.2 | 245.0818 |
| | | | $[M+H]^+$ | 291.0866 | 291.0863 | 1.1 | 12.6 | | 123.0449<br>139.0387 |
| ♣ | Vanillin | $C_8H_8O_3$ | $[M-H]^-$ | 151.0396 | 151.0401 | 3.0 | 3.1 | 10.0 | 136.0158 |
| | | | $[M+H]^+$ | 153.0536 | 153.0546 | 6.6 | 51.5 | | 137.0252 |
| ♣ | *p*-coumaric acid | $C_9H_8O_3$ | $[M-H]^-$ | 163.0396 | 163.0401 | 3.1 | 3.6 | 10.3 | 119.0479 |
| | | | $[M+H]^+$ | 165.0544 | 165.0546 | 1.2 | 4.8 | | 147.0436<br>119.0492 |
| ♣ | Ferulic acid | $C_{10}H_{10}O_4$ | $[M-H]^-$ | 193.0506 | 193.0506 | 0.1 | 6.2 | 11.5 | 178.0273<br>134.0361 |
| | | | $[M+H]^+$ | 195.0652 | 195.0652 | 0.1 | 7.6 | | 177.0541<br>149.0593 |
| ▬ | Rutin | $C_{27}H_{30}O_{16}$ | $[M-H]^-$ | 609.1470 | 609.1461 | -1.5 | 2.1 | 11.6 | - |
| | | | $[M+H]^+$ | 611.1606 | 611.1606 | 0.1 | 52.5 | | 303.0499 |
| ♣ | Sinapinic acid | $C_{11}H_{12}O_5$ | $[M-H]^-$ | 223.0620 | 223.0612 | -3.4 | 4.8 | 11.7 | 193.0147<br>208.0381 |
| | | | $[M+H]^+$ | 225.0759 | 225.0758 | -4.5 | 6.5 | | 207.0652 |
| ♣ | Ellagic acid | $C_{14}H_6O_8$ | $[M-H]^-$ | 301.0005 | 300.9990 | -5.1 | 3.8 | 11.7 | - |
| | | | $[M+H]^+$ | 303.0135 | 303.0135 | -0.1 | 2.9 | | - |
| ♣ | 3-hydroxycinnamic acid | $C_9H_8O_3$ | $[M-H]^-$ | 163.0402 | 163.0401 | -0.9 | 6.1 | 11.8 | 119.0479 |
| | | | $[M+H]^+$ | 165.0545 | 165.0546 | 0.1 | 6.1 | | - |
| ■ | Taxifolin | $C_{15}H_{12}O_7$ | $[M-H]^-$ | 303.0510 | 303.0510 | 0.2 | 5.1 | 11.9 | 607.1061 |
| | | | $[M+H]^+$ | 305.0640 | 305.0656 | 3.2 | 71.4 | | 231.0647<br>259.0598 |
| ♣ | Benzoic acid | $C_7H_6O_2$ | $[M-H]^-$ | 121.0292 | 121.0295 | 2.4 | 11.8 | 12.5 | 96.0593 |
| | | | $[M+H]^+$ | 123.0444 | 123.0441 | -2.5 | 9.1 | | 98.9751<br>105.0397 |
| ◆ | Narirutin | $C_{27}H_{32}O_{14}$ | $[M-H]^-$ | 579.1712 | 579.1719 | 1.3 | 6.0 | 12.8 | 1159.3604 |
| | | | $[M+H]^+$ | 581.1881 | 581.1865 | -1.6 | 36.9 | | 273.0739 |
| ◆ | Naringin | $C_{27}H_{32}O_{14}$ | $[M-H]^-$ | 579.1737 | 579.1719 | -3.1 | 9.0 | 13.3 | 1159.3604 |
| | | | $[M+H]^+$ | 581.1871 | 581.1865 | -1.0 | 64.8 | | 273.0744 |
| ▬ | Quercetin-3-*O*-glucose-6''-acetate | $C_{23}H_{22}O_{13}$ | $[M-H]^-$ | 505.0986 | 505.0988 | 0.4 | 5.1 | 13.5 | - |
| | | | $[M+H]^+$ | 507.1133 | 507.1133 | 0.0 | 36.0 | | 303.0499 |
| ▬ | Myricetin | $C_{15}H_{10}O_8$ | $[M-H]^-$ | 317.0302 | 317.0303 | 0.4 | 3.6 | 14.1 | - |
| | | | $[M+H]^+$ | 319.0444 | 319.0448 | 1.3 | 4.2 | | 273.0668 |
| ● | Neohesperidin | $C_{28}H_{34}O_{15}$ | $[M-H]^-$ | 609.1817 | 609.1825 | 1.3 | 10.2 | 14.2 | - |
| | | | $[M+H]^+$ | 611.1973 | 611.1970 | -0.4 | 54.7 | | - |
| ▬ | Kaempferol | $C_{15}H_{10}O_6$ | $[M-H]^-$ | 285.0400 | 285.0405 | 1.6 | 1.2 | 16.8 | - |
| | | | $[M+H]^+$ | 287.0550 | 287.0550 | -0.1 | 9.1 | | - |
| ▬ | Quercetin | $C_{15}H_{10}O_7$ | $[M-H]^-$ | 301.0348 | 301.0354 | 2.0 | 9.2 | 16.8 | - |
| | | | $[M+H]^+$ | 303.0495 | 303.0499 | -0.4 | 17.7 | | - |
| ♣ | *trans*-cinnamic acid | $C_9H_8O_2$ | $[M-H]^-$ | 147.0440 | 147.0452 | 2.7 | 3.1 | 16.9 | - |
| | | | $[M+H]^+$ | 149.0596 | 149.0597 | 0.1 | 5.8 | | 103.0543 |
| □ | Laricitrin | $C_{16}H_{12}O_8$ | $[M-H]^-$ | 331.0468 | 331.0459 | -2.6 | 11.6 | 17.1 | - |
| | | | $[M+H]^+$ | 333.0608 | 333.0605 | -0.9 | 18.2 | | - |
| ◆ | Poncirin | $C_{28}H_{34}O_{14}$ | $[M-H]^-$ | 593.1902 | 593.1876 | -4.4 | 8.1 | 17.2 | - |
| | | | $[M+H]^+$ | 595.2026 | 595.2021 | -0.7 | 38.6 | | - |
| ◆ | Naringenin | $C_{15}H_{12}O_5$ | $[M-H]^-$ | 271.0616 | 271.0612 | 1.6 | 3.1 | 18.7 | - |
| | | | $[M+H]^+$ | 273.0753 | 273.0758 | 1.7 | 18.0 | | - |
| □ | Apigenin | $C_{15}H_{10}O_5$ | $[M-H]^-$ | 269.0458 | 269.0455 | -0.8 | 6.9 | 18.9 | - |
| | | | $[M+H]^+$ | 271.0596 | 271.0601 | 2 | 12.9 | | - |
| □ | Luteolin | $C_{15}H_{10}O_6$ | $[M-H]^-$ | 285.0401 | 285.0405 | 1.3 | 2.3 | 19.3 | - |
| | | | $[M+H]^+$ | 287.0552 | 287.0550 | -0.5 | 6.8 | | - |
| ▬ | Isorhamnetin | $C_{16}H_{12}O_7$ | $[M-H]^-$ | 315.0512 | 315.0510 | -0.5 | 3.3 | 19.9 | 300.0277 |
| | | | $[M+H]^+$ | 317.0657 | 317.0656 | -0.3 | 11.6 | | 287.0551 |
| □ | Chrysin | $C_{15}H_{10}O_4$ | $[M-H]^-$ | 253.0508 | 253.0506 | -0.8 | 1.8 | 24.2 | - |
| | | | $[M+H]^+$ | 255.0654 | 255.0652 | -0.9 | 2.5 | | - |
| ◆ | Pinocembrin | $C_{15}H_{12}O_4$ | $[M-H]^-$ | 255.0675 | 255.0663 | -4.8 | 6.3 | 24.7 | - |
| | | | $[M+H]^+$ | 257.0798 | 257.0808 | 4.1 | 96.2 | | - |
| □ | Galangin | $C_{15}H_{10}O_5$ | $[M-H]^-$ | 269.0445 | 269.0455 | 3.8 | 4.9 | 25.0 | - |
| | | | $[M+H]^+$ | 271.0601 | 271.0601 | -0.1 | 14.4 | | - |
| ▬ | Kaempferide | $C_{16}H_{12}O_6$ | $[M-H]^-$ | 299.0556 | 299.0561 | 1.5 | 2.3 | 25.3 | 284.0310 |
| | | | $[M+H]^+$ | 301.0707 | 301.0707 | 0.1 | 4.5 | | - |
| ◆ | Pinocembrin-7-methyleter | $C_{16}H_{14}O_4$ | $[M-H]^-$ | 269.0816 | 269.0819 | 1.2 | 4.1 | 30.9 | - |
| | | | $[M+H]^+$ | 271.0965 | 271.0965 | 0.1 | 4.9 | | 167.0343 |

[a] Black clover, phenolic acids and related compounds; black rectangle, flavonol; black diamond, flavanone; white square, flavone; black triangle, flavanol; black square, dihydroflavonol; black circle, dihydrochalcone.

obtained from Lab-Scan (Gliwice, Sowinskiega, Poland). To prepare the mobile phases, we used acetonitrile (ACN) and acetic acid, and they were purchased from Lab-Scan (Dublin, Ireland) and Panreac, respectively. All of the solvents used were of HPLC grade, and they were used as received. Doubly deionized water with a conductivity of 18.2 MΩ was obtained by using a Milli-Q system (Millipore, Bedford, MA).

Standards of phenolic compounds (vanillin, vanillic acid, homovanillic acid, ferulic acid, 4-hydroxybenzoic acid, benzoic acid, *trans*-cinnamic acid, syringic acid, caffeic acid, gentisic acid, *p*-coumaric acid, protocatechuic acid, sinapinic acid, gallic acid, ellagic acid, chlorogenic acid, 3-hydroxycinnamic acid, taxifolin, quercetin, luteolin, kaempferol, naringenin, apigenin, myricetin, laricitrin, galangin, chrysin, pinocembrin, pinocembrin-7-methyl ether, poncirin, naringin, kaempferide, quercetin-3-*O*-glucose-6′′-acetate, isorhamnetin, catechin, narirutin, rutin, epicatechin, neohesperidin, myrtillin, kuromanin, cyanin, cyanidin-3-*O*-β-glucopyranoside, cyanidin-3-*O*-β-galactopyranoside, syringetin, cyanidin-3-*O*-2′′-*O*-β-xylopyranosyl-β-glucopyranoside, delphinidin-3-*O*-2′′-*O*-β-xylopyranosyl-β-glucopyranoside, delphinidin-3-*O*-β-glucopyranoside, and delphinidin-3-*O*-6′′-*O*-α-ramnopyranosyl-β-glucopyranoside) were mostly purchased from Extrasynthese (Lyon, France), Sigma-Aldrich (St. Louis, MO), and Fluka (St. Louis, MO). All of them were used during the preliminary studies.

Taking into account the previously published literature about the determination of phenols from avocado in particular, or fruit, in general,[17,22−26] the availability of standards, and our preliminary studies (checking the phenols that were present in every avocado variety we had), we tried to prepare a mixture to carry out the method optimization. The idea was to create a representative mix that could be useful regardless of the avocado variety under study. Moreover, the standard mix was created to cover a wide range of polarities and molecular weights mimicking as closely as possible what we could find in avocado samples.

Finally, this mixture was made with 39 phenolic compounds belonging to phenolic acids or related compounds, flavones, flavanones, flavonols, dihydrochalcones, and dihydroflavonols (see Table 1). We used the same amount of each standard, and we added the necessary volume of methanol to have stock solutions containing 100 ppm of each compound.

All stock solutions, samples, solvents, and reagents were filtered with a 5 μm membrane filter (Millipore, Bedford, MA) before separation or injection in the instrument.

**Avocado Samples Used in This Study.** We used three varieties of avocado: Hass, Lamb−Hass, and Rugoro. For each variety, we used the pulp of three to four pieces of fruit, which were frozen to be further freeze-dried. Many studies advise starting from samples freeze-dried, because their subsequent conservation is easier.[19,22,27]

**Extraction of Phenolic Compounds from Avocado.** The preliminary studies led us to use acetone, methanol, ethanol, and ethyl acetate, as the most proper solvents for the extraction of the secondary metabolites under study from avocado samples.

We prepared four extracts starting with 4 g of the freeze-dried (and homogenized) sample and using 40 mL of pure methanol, ethanol, acetone, and ethyl acetate. We put the sample and the solvent inside a falcon tube, and they were shaken in a vortex during 30 min. The supernatants were taken and centrifuged at 4500 rpm for 10 min. After that, the supernatants were evaporated to dryness and redissolved in 1 mL of methanol. Finally, the extracts were preconcentrated to 100 μL of methanol.

Our aim was to determinate which one of the four selected solvents was able to extract more compounds in a higher concentration, using the same amount of sample and the same volume of solvent. The three varieties of avocado under studied were extracted by using the different solvents.

**HPLC Analyses.** An Agilent 1200-RRLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, an autosampler, a binary pump, and a UV−Vis detector was used for the chromatographic determination. Phenolic compounds were separated by using a reverse phase $C_{18}$ analytical column (Zorbax Eclipse Plus, 4.6 × 150 mm, 1.8 μm particle size). Mobile phases A and B consist of water with 0.5% acetic acid and ACN, respectively. Several gradients (A−D) were used to achieve the best separation among the 39 compounds belonging to our standard mix; the gradients changed according to the conditions described in Table 2.

**Table 2. Different Gradients Used during the Optimization of the Chromatographic Method**

| | | gradient | |
|---|---|---|---|
| method | minute | phase A (%) | phase B (%) |
| A | 0 | 99 | 1 |
| | 55 | 0 | 100 |
| | 57.5 | 99 | 1 |
| | 60 | 99 | 1 |
| B | 0 | 99 | 1 |
| | 55 | 40 | 60 |
| | 57 | 0 | 100 |
| | 60 | 99 | 1 |
| C | 0 | 99 | 1 |
| | 20 | 90 | 10 |
| | 55 | 40 | 60 |
| | 57 | 0 | 100 |
| | 60 | 99 | 1 |
| D | 0 | 99 | 1 |
| | 20 | 90 | 10 |
| | 50 | 60 | 40 |
| | 55 | 40 | 60 |
| | 57 | 0 | 100 |
| | 60 | 99 | 1 |

The flow rate used was 1.60 mL/min. The room temperature was kept at 20 °C. A volume of 10 μL of the avocado extracts or stock standard solutions was injected. The compounds separated were monitored in sequence, first with DAD over the range of 190−600 nm to achieve spectral data and then with an ESI-TOF mass spectrometry detector. Peak identification was done bearing in mind migration time, spectral data, and ESI-TOF MS information obtained from real samples and standards and also with spiked real samples at different concentration levels.

**Mass Spectrometry.** The HPLC system was coupled to a Micro-TOF (Bruker Daltonik, Bremen, Germany) using an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA). The TOF analyzer provides greatly improved mass resolution (10000−15000 at $m/z$ 300) and significantly higher sensitivity and accuracy in the acquisition of full-fragment spectra compared with traditional instruments.

As mentioned before, the TOF mass spectrometer was equipped with an electrospray interface (ESI) operating in negative and positive ion polarity (sequential analyses) using a capillary voltage of ±4.5 kV. The flow rate used in the chromatographic method, 1.6 mL/min, was too high for achieving stable electrospray ionization (ESI) (maximum flow rate is around 1 mL/min); therefore, it was necessary to use a flow divisor of 1:6. In that way, the flow delivered into the mass spectrometer was reduced to 0.3 mL/min, low enough to avoid the introduction of humidity into the system. The other optimum values of the ESI-MS parameters, according to this flow, were as follows: drying gas temperature, 190 °C; drying gas flow, 9 L/min; and nebulizer gas pressure, 2 bar.

The polarity of ESI and all of the parameters of MS detector were optimized using the height of the MS signal for the 39 phenolic compounds included in our standard mix as analytical parameter.

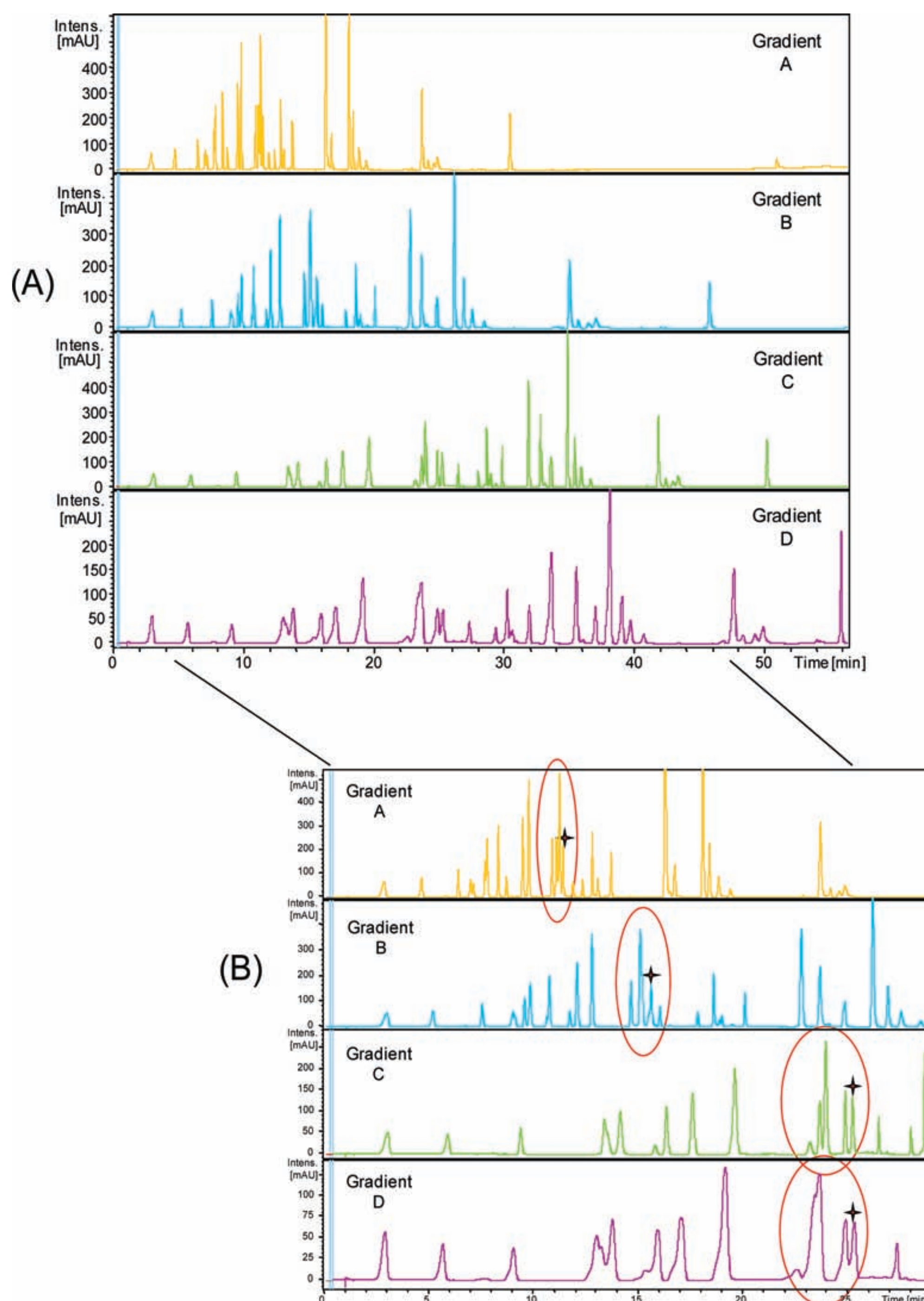


**Figure 1.** (A) Different profiles obtained for the standard mix containing 39 phenolic compounds at 280 nm by using several gradients during the method optimization. (B) Detail of the separation achieved between 0 and 28 min when gradients A−D were used. The most critical area is highlighted, and the asterisk identifies the same compound in all cases.

The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltonik), which provided a list of possible elemental formulas by using the SmartFormula Editor tool. The SmartFormula Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theorical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula. The smaller the sigma value and the error, the better the fit; therefore, for routine screening an error of 5 ppm and a threshold sigma value of 0.05 are generally considered to be appropriate. Even with very high mass accuracy (<1 ppm), many chemically possible formulas are obtained depending on the mass regions considered. Therefore, high mass accuracy (<1 ppm) alone is not sufficient to exclude enough candidates with complex elemental compositions. The use of isotopic abundance patterns removes >95% of false candidates.

External calibration was performed with sodium formate clusters by using a solution containing 5 mM sodium hydroxide and 0.2% formic acid in water/isopropanol 1:1 v/v. The calibration solution was injected at the beginning of the run, and all of the spectra were calibrated prior to phenol identification.

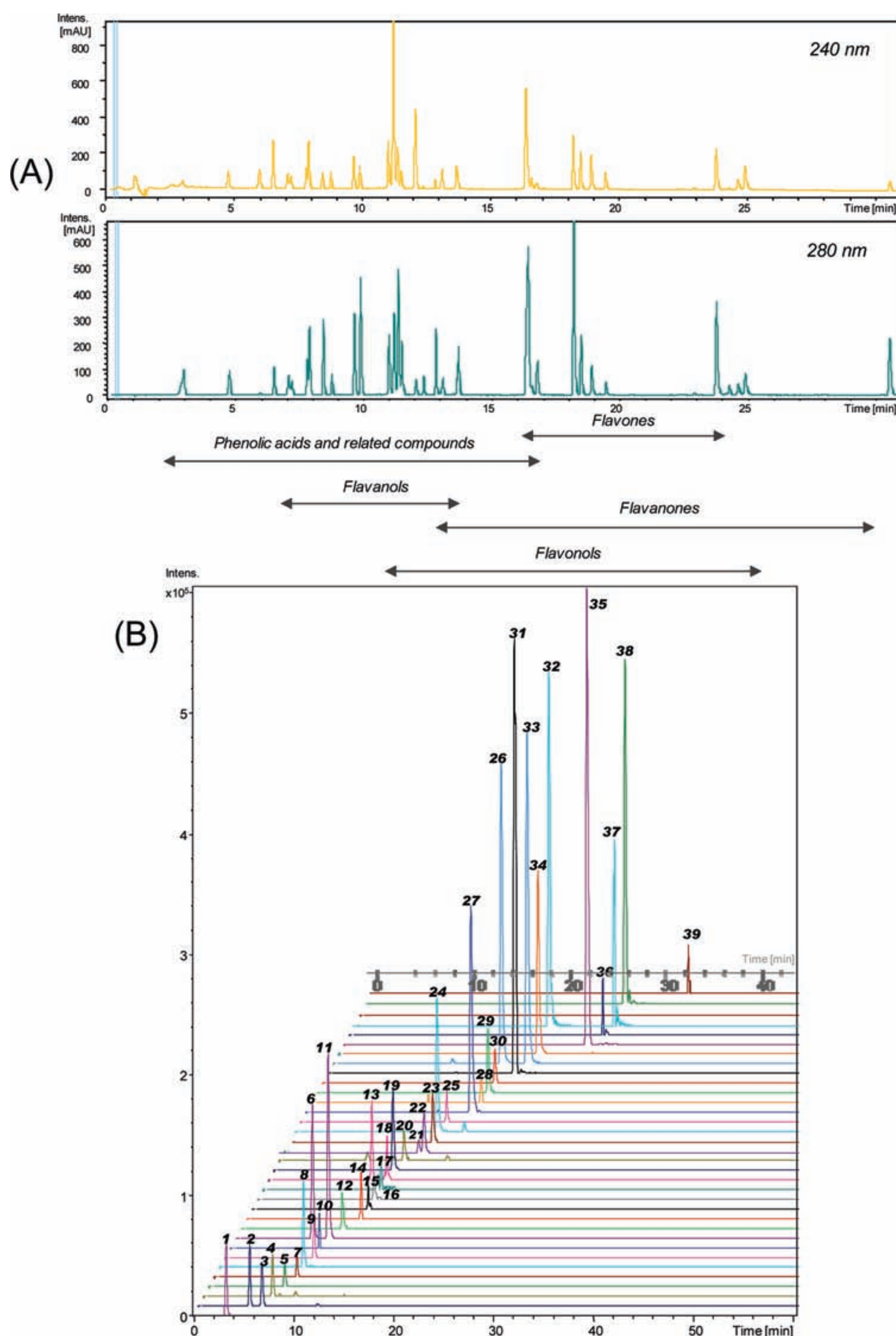


**Figure 2.** (A) UV profiles (240 and 280 nm) of the standard mixture of 39 phenolic compounds. Areas of elution of phenolic acids and related compounds, flavones, flavanols, flavanones, and flavonols are defined. (B) Extract ion chromatograms (EICs) obtained when the optimum parameters for the separation and detection were used. Peaks: 1, gallic acid; 2, protocatechuic acid; 3, gentisic acid; 4, 4-hydroxybenzoic acid; 5, chlorogenic acid; 6, catechin; 7, vanillic acid; 8, caffeic acid; 9, syringic acid; 10, homovanillic acid; 11, epicatechin; 12, vanillin; 13, *p*-coumaric acid; 14, ferulic acid; 15, ellagic acid; 16, sinapinic acid; 17, rutin; 18, 3-hydroxycinnamic acid; 19, taxifolin; 20, benzoic acid; 21, narirutin; 22, naringin; 23, quercetin-3-*O*-gluc-6′′-acet; 24, myricetin; 25, neohesperidin; 26, *trans*-cinnamic acid; 27, quercetin; 28, kaempferol; 29, laricitrin; 30, poncirin; 31, naringenin; 32, apigenin; 33, luteolin; 34, isorhamnetin; 35, chrysin; 36, pinocembrin; 37, galangin; 38, kaempferide; 39, pinocembrin-7-methyl ether.

## RESULTS AND DISCUSSION

**HPLC-ESI-TOF MS Method Optimization.** The solution containing the 39 phenolic compounds included in Table 1 was used to optimize both the chromatographic and MS conditions. Initially, the chromatographic conditions were optimized according to the following criteria: chromatographic behavior (retention time, which depends on the polarity of the compounds under study), sensitivity, analysis time, and peak shape.

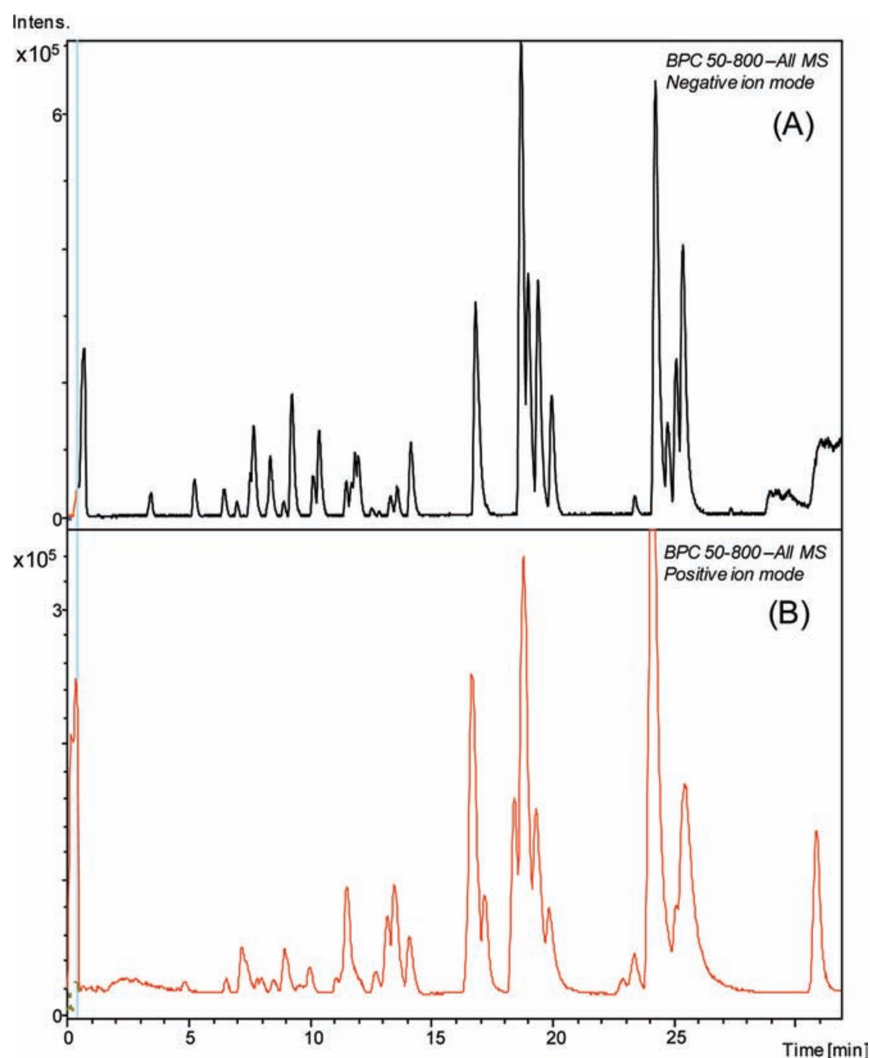


**Figure 3.** BPCs obtained in negative (A) and positive (B) ion polarity for the standard mixture.

The quality of any chromatographic separation depends on the characteristics of the compounds to be separated and their interactions with the column and solvent. A profiling method for phenolic compounds requires uniform separation across the range of polarities, from the most polar (hydroxybenzoic and hydroxycinnamic acids) to the least polar (aglycones and polymethoxylated flavonoids) compounds.

For the chromatographic separation we must select the proper chromatographic parameters (mobile phases, temperature, column type, chromatographic modes, etc.) to get the best response, with the best possible resolution among the analytes to be separated. Considering the literature previously published about the determination of this kind of compounds from different fruits and plants, our own experience, and the results we got during the preliminary studies, water + acetic acid (0.5%) and acetonitrile were used as mobile phases.[2,14,15] The different profiles obtained at 280 nm by using gradients A−D are shown in Figure 1. In Figure 1A, we can see that the method which provided the fastest separation was gradient A, because the composition of the mobile phase (regarding percentage of B (ACN)) is increasing more drastically. At that moment, we wanted to determine that gradient A was able to give us adequate resolution or if perhaps one of the other methods could provide better resolution. The most critical area of the chromatogram is the one that the reader can see more in detail in Figure 1B. The red asterisk marks the same compound in every case to facilitate the comparison. Gradient D gave us the longest analysis time without giving better resolution. Gradients B and C gave similar results, although gradient C is the method that provides better resolution in the critical area; however, the efficiency of the separation and the number of theoretical plates between 5 and 20 min were not very satisfactory. Considering the fact that detection was made not only by DAD but also with the powerful TOF MS analyzer, we considered as a compromise solution to use gradient A as optimum, because it provided reasonably good resolution, excellent peak shape, and short analysis time and was a potent "profiling method" able to separate and detect as many of the components as possible in a single extract of a food material (changing the polarity of the mobile phase in the whole possible range, 1−100% B).

The optimization of ESI-TOF MS conditions was made by direct infusion experiments of the standard mixture containing 39 phenolic compounds; we optimized source, transfer, and detection parameters looking for the maximum sensitivity with the highest possible resolution. All of the optimum parameters have been mentioned previously (see Mass Spectrometry under Materials and Methods).

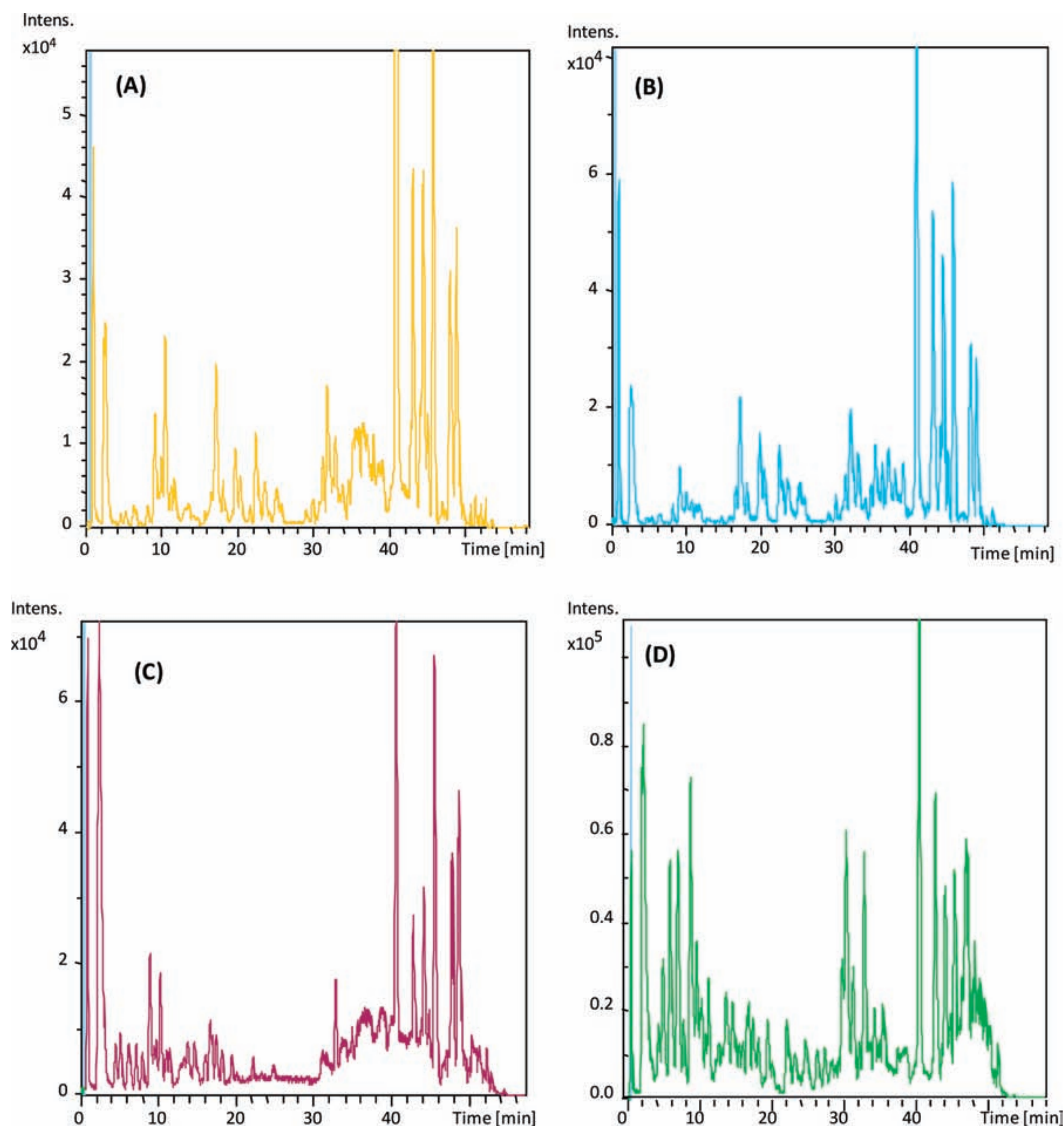


**Figure 4.** Comparison of the base peak chromatograms of extracts from Hass variety avocado fruit prepared by using (A) acetone, (B) ethyl acetate, (C) ethanol, and (D) methanol as extractant agent.

Figure 2 shows the UV profiles at 240 and 280 nm and the extract ion chromatograms (EICs) that compose the base peak chromatogram (BPC) (in negative polarity) of the standard mixture. We indicate as well in Figure 2 the different areas of elution depending on the family of phenol (phenolic acids and related compounds, flavones, flavanols, etc.).

We made the analyses in negative and positive ion mode because we could obtain additional information using both polarities and we could corroborate/confirm the identity of the compounds under study. Figure 3 shows a comparison of the BPC obtained for the standard mix in positive and negative polarity. We can see that in negative polarity, the response factor of some compounds (phenolic acids and flavones, for example) is higher than in positive mode, because not all of the compounds are equally ionized in both polarities. For instance, gallic, protocatechuic, and gentisic acids could not be detected properly in positive polarity (although the concentration was exactly the same), but the opposite happened for some other compounds. That is why we decided to carry out the detection in both polarities in every case, to have complementary information; this could be particularly important in the analysis of real avocado samples.

Table 1 summarizes information in positive and negative polarity of the standard mix containing 39 phenolic compounds, including their formula, selected ion, $m/z$ experimental and calculated, error (in ppm), mSigma value, migration time, and fragments in source or other signals in MS.

**Extraction of the Compounds under Study from Avocado Matrix.** The initial extraction procedure is generally aimed at maximizing the amount and concentration of the compounds of interest. For that reason, we could say that the extraction can be considered as a very important step in this kind of study.

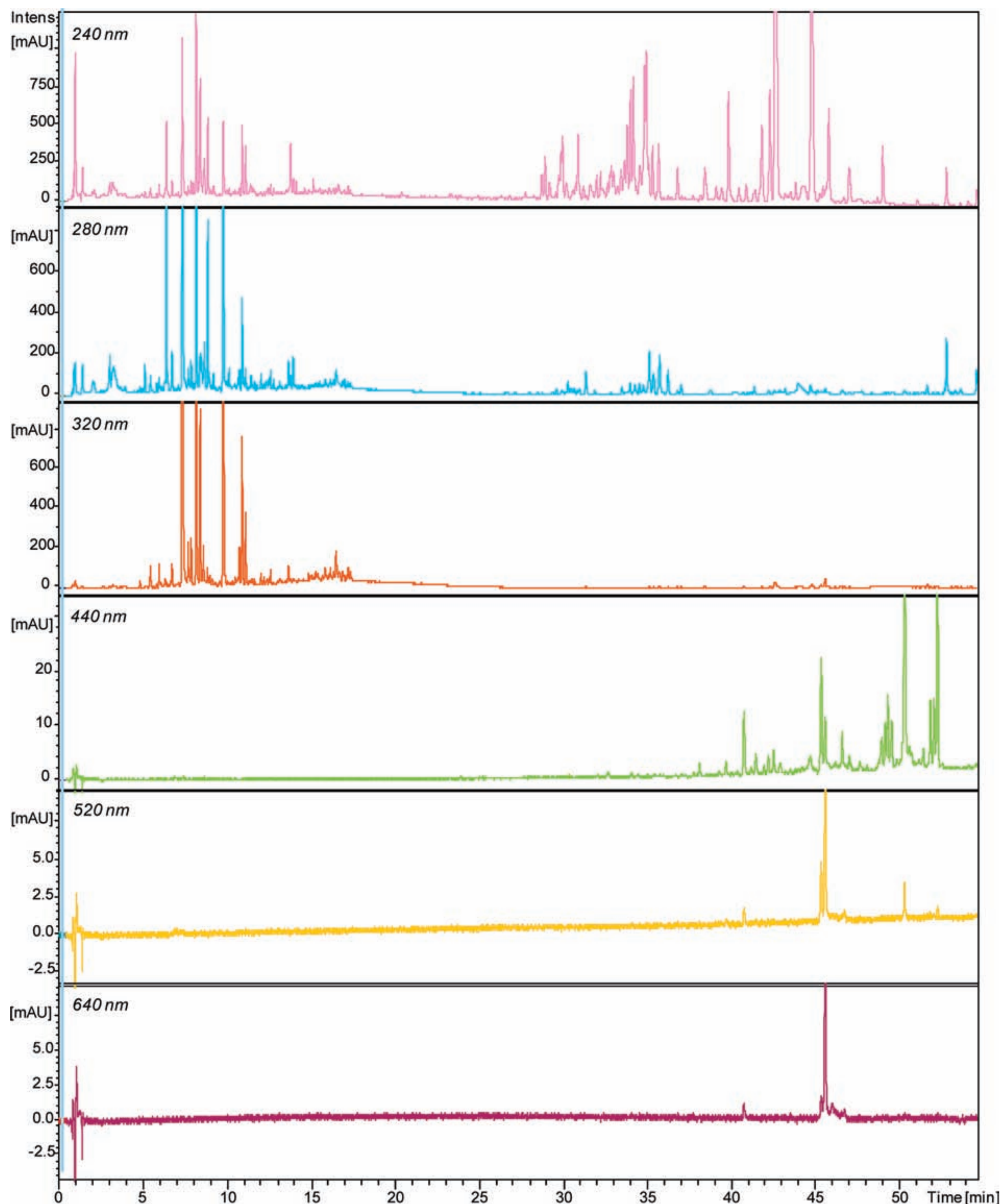


**Figure 5.** UV−Vis profiles at different wavelengths of a methanolic extract from a Hass avocado sample.

To carry out the optimization of the extraction protocol, we kept in mind that the solubility of phenols is controlled by the polarity of the solvent used and the degree of polymerization of phenols, as well as the interaction of phenolic compounds with other plant-fruit constituents, such as carbohydrate and proteins.[2,28] The solvents used more often in this kind of study are methanol, ethanol, acetone, water, ethyl acetate, and, to a lesser extent, propanol, dimethylformamide, dimethyl sulfoxide, and their combinations.[29] The preliminary studies led us to use acetone, methanol, ethanol, and ethyl acetate as solvents for the extraction, because their polarities are quite similar (around 4.4−5.2).

In Figure 4 we can see the profiles (BPC in negative polarity) of the four extracts obtained with the different solvents used (acetone, methanol, ethanol, and ethyl acetate). The comparison was made by taking into account the total integrated area, the number of peaks, and the number of molecular features found by the software Data Analysis in each case by using exactly the same

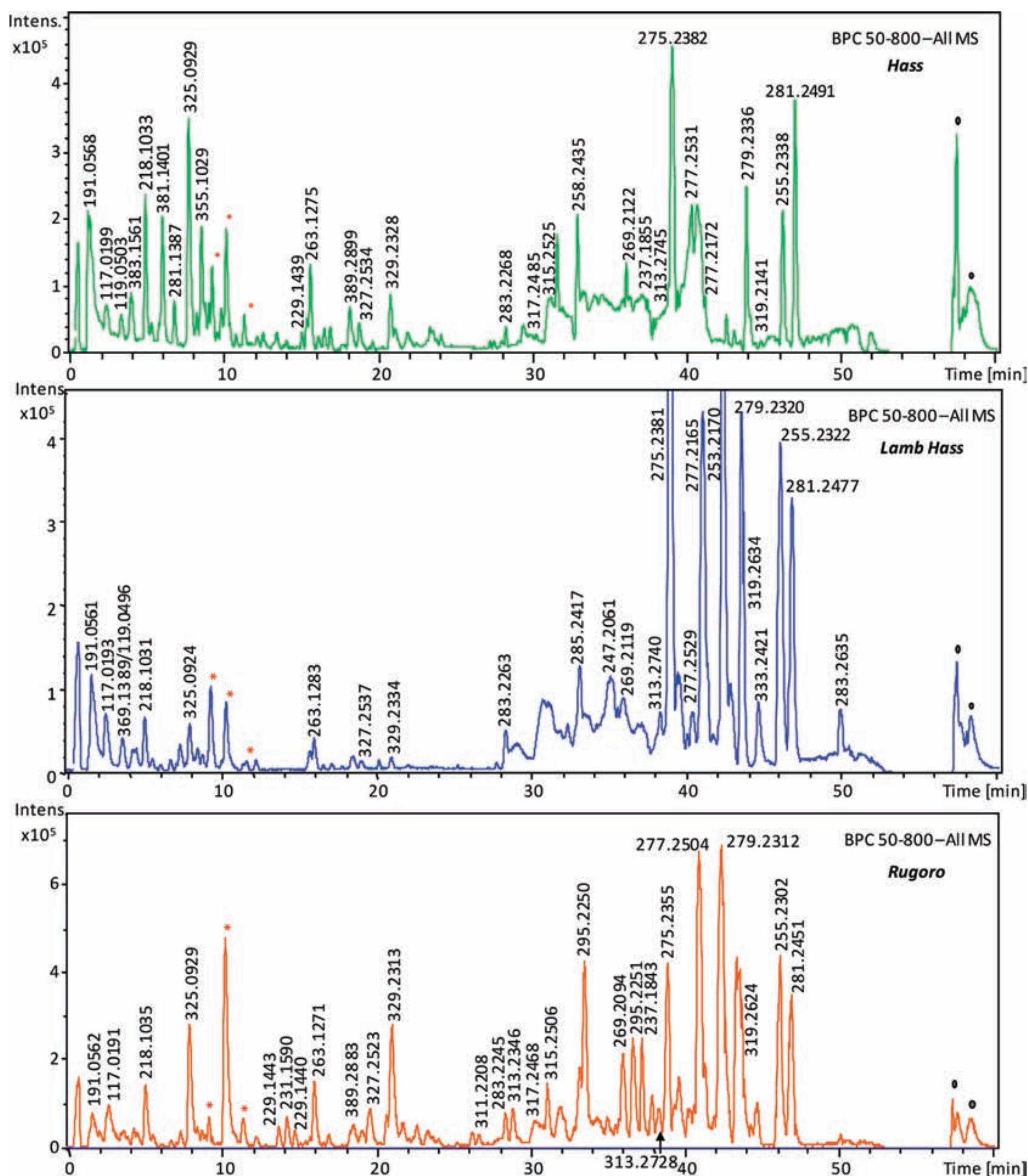


Figure 6. BPCs of the methanolic extract obtained from Hass, Lamb–Hass, and Rugoro varieties analyzed under optimal conditions. (Only peaks with relevant intensity are shown.) Asterisks indicate compounds for which the identity was corroborated with standards. Circles indicate peaks that appeared when blanks were analyzed (do not consider).

parameters as far as S/N threshold, area threshold, intensity threshold correlation coefficient threshold, and minimum compound length are concerned. MeOH gave the richest profile in terms of number of peaks extracted and their concentration; the difference can be seen clearly between 2 and 25 min. The extracts obtained with acetone and ethanol were quite similar, although the acetone extracted a greater number of compounds in comparison with ethanol. Although the four solvents used were able to extract the phenolic compounds, methanol extracts presented the best recovery. Moreover, methanol gave us very clean extracts, which could be preconcentrated more easily. For this reason, we will show only the profiles for the extracts of avocado obtained with methanol.

**Potential of the Described Method.** The BPC of the methanolic extract from avocado was already shown in Figure 4D (Hass variety), but we think that it is very interesting to show and compare the UV–Vis profiles at 240, 280, 320, 440, 520, and 640 nm (see Figure 5). The mentioned wavelengths were especially selected, because, for instance, 240 and 280 nm have been widely used to determine different families of phenolic compounds, 280 nm being particularly useful for determining phenolic acids; 320 nm (approximate) is a very suitable wavelength when different subclasses of flavonoids have to be determined, and 440 and 520 nm (approximate) are recommended when determination of anthocyanins needs to be achieved. We decided to include the channel of 640 nm as well to facilitate the possible detection of pigments, which were supposed to be extracted by MeOH, because the extracts were strongly colored.

DAD together with MS contributes to the whole picture of the sample with structural information (phenolic compound family).

Table 3. Phenolic Secondary Metabolites Found in the Methanolic Extracts Obtained from the Three Different Varieties of Avocado under Study (Organized by Upward Retention Time) for Which the Identity Was Corroborated by Standards[a]

| | | | | | variety of avocado fruit | | |
|---|---|---|---|---|---|---|---|
| compound | formula | *m/z* exptl | *m/z* theor | retention time (min) | Rugoro | Lamb−Hass | Hass |
| protocatechuic acid | $C_7H_6O_4$ | 153.0196 | 153.0193 | 5.3 | X | X | X |
| gentisic acid | $C_7H_6O_4$ | 153.0201 | 153.0193 | 6.4 | X | X | − |
| 4-hydroxybenzoic acid | $C_7H_6O_3$ | 137.0242 | 137.0244 | 6.9 | X | − | − |
| chlorogenic acid | $C_{16}H_{18}O_9$ | 353.0879 | 353.0878 | 7.5 | X | − | − |
| catechin | $C_{15}H_{14}O_6$ | 289.0701 | 289.0718 | 7.7 | X | X | X |
| caffeic acid | $C_9H_8O_4$ | 179.0353 | 179.0350 | 8.3 | X | X | − |
| epicatechin | $C_{15}H_{14}O_6$ | 289.0718 | 289.0718 | 9.3 | X | X | X |
| vanillin | $C_8H_8O_3$ | 151.0396 | 151.0401 | 9.9 | X | − | − |
| *p*-coumaric acid | $C_9H_8O_3$ | 163.0400 | 163.0401 | 10.3 | X | X | X |
| ferulic acid | $C_{10}H_{10}O_4$ | 193.0494 | 193.0506 | 11.4 | X | X | X |
| sinapinic acid | $C_{11}H_{12}O_5$ | 223.0616 | 223.0612 | 11.6 | X | X | X |
| benzoic acid | $C_7H_6O_2$ | 121.0295 | 121.0295 | 12.5 | − | − | X |
| *trans*-cinnamic acid | $C_9H_8O_2$ | 147.0454 | 147.0452 | 16.8 | X | X | X |
| laricitrin | $C_{16}H_{12}O_8$ | 331.0452 | 331.0459 | 17.1 | X | − | − |
| naringenin | $C_{15}H_{12}O_5$ | 271.0618 | 271.0612 | 18.7 | X | − | − |
| chrysin | $C_{15}H_{10}O_4$ | 253.0493 | 253.0506 | 24.3 | X | − | − |
| kaempferide | $C_{16}H_{12}O_6$ | 299.0543 | 299.0561 | 25.3 | X | − | − |

[a] Error (ppm) and mSigma values were lower than 3.8 and 17.1 in every case. X means that the compound was present in the avocado sample. − means that the compound was not present in the avocado sample.

Taking together the information provided by both detectors represents a flexible tool for explorative studies and could be very useful in any metabolic profiling study.

The aim of this section is to point out that, apart from the phenols we were interested in, some other constituents of avocado fruit could be separated and determined by our method. Taking advantage of the saved information, we could enlarge our knowledge (quite limited so far) about the composition of this important fruit.

**Application of Method to the Analysis of Avocado Samples.** We proceeded to analyze three different varieties of avocado. Figure 6 shows the BPC in negative polarity of the methanolic extracts obtained for the three varieties of avocado under study.

The Rugoro variety contained the largest number of compounds for which identity was confirmed with standards, as demonstrated in Table 3. This table includes information concerning the molecular formula (the compounds are in elution order), *m/z* experimental and *m/z* theoretical (in negative polarity), and retention time. We indicate in the table as well in which varieties the compounds were found. Considering the 39 phenolic compounds included in the standard mixture used for the optimization of the method, we identified 17 phenols in our avocado samples. The 17 compounds were not found in the 3 varieties of avocado; for instance, in the Rugoro variety we could identify unequivocally 16 phenols, namely, protocatechuic acid, gentisic acid, 4-hydroxybenzoic acid, chlorogenic acid, catechin, caffeic acid, epicatechin, vanillin, *p*-coumaric acid, ferulic acid, sinapic acid, *trans*-cinnamic acid, laricitrin, naringenin, chrysin, and kaempferide.

Apart from the compounds that we could identify with the help of commercial standards, there were other constituents of avocado that we could detect in the chromatograms. Using the potential of the analyzer that we were using, we could achieve a tentative identification, determining the *m/z* experimental, the possible molecular formula, and even the name(s) of possible candidate(s). Figure 6 shows the *m/z* experimental signal found for some of the most intense compounds of the BPC, and Table 4 summarizes that information, including the list of possible molecular formulas achieved by SmartEditor (upward order of sigma), a list of possible compounds, and MS fragments in source for Rugoro, Hass, and Lamb−Hass varieties, respectively.

We tried to achieve a reliable identification of every compound detected within the chromatographic run using negative and positive ion polarities. Indeed, using this information (together with the fragmentation in source), we could corroborate the tentative identification.

A large number of compounds were identified in negative polarity in all samples of avocado, specifically 29 in Rugoro, 27 in Hass, and 22 in Lamb−Hass. Positive mode made it possible to corroborate the identity of 13 compounds in Rugoro, 14 compounds in Hass, and other 12 in Lamb−Hass samples. Those compounds belong to alkanols (1,2,4-trihydroxynonadecane, (2*S*,4*S*)-2,4-dihydroxyheptadec-16-enyl acetate, etc.), furan ring containing derivatives (2-(1-pentadecenyl)furan and pentadecylfuran), carboxylic acids (quinic acid, succinic acid, and methylmalonic acid), saturated and unsaturated fatty acids (2-methoxy-5*Z*-hexadecenoic acid, 8-hydroxylinoleic acid, palmitic acid, oleic acid, dodecanoic acid, etc.), isoprenoid plant hormones (abscisic acid), and sesquiterpenoids (centarol, daucol), among others.

The three avocado varieties under study contained common compounds, such as quinic acid, succinic acid, pantothenic acid, *p*-coumaroyl-D-glucose, abscisic acid, pentadecylfuran, avocado furan, and oleic acid. However, some compounds were present in only one of the varieties analyzed, being potential varietal markers. It is necessary to continue the current study increasing the number of varieties analyzed to be able to ensure that a particular compound is a varietal marker; $MS^n$ analyses would be advisable as well.

Table 4. Tentative Identification of Compounds Found in the Methanolic Extracts from Hass, Lamb−Hass, and Rugoro Avocado Samples with Considerable Intensity in the BPC[a]

| negative ion polarity | | | | | | | | positive ion polarity | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | variety | | | | | |
| *m*/*z* exptl | retention time (min) | possibilities in Smart Editor-Molecular formula (upward order of sigma) | possible compounds (for the first formula) | Hass | Lamb−Hass | Rugoro | MS fragments in source | *m*/*z* exptl | MS fragments in source |
| 191.0561 | 1.5 | $C_7H_{12}O_6$ | quinic acid | X | X | X | – | 193.0716 | – |
| 117.0193 | 2.5 | $C_4H_6O_4$ | succinic acid<br>methylmalonic acid | X | X | X | – | 119.0398 | – |
| 119.0496 | 3.5 | $C_8H_8O_1$ | isocoumaran | X | X | – | – | 121.0692 | 95.0498 |
| 383.1561 | 4.0 | $C_{15}H_{28}O_{11}$<br>$C_{14}H_{22}N_7O_6$ | – | X | – | – | – | – | – |
| 218.1031 | 5.0 | $C_9H_{17}N_1O_5$<br>$C_8H_{10}N_8$ | pantothenic acid (vitamin $B_5$) | X | X | X | 146.0814 | 220.1174 | 98.9770<br>160.0822 |
| 281.1387 | 6.8 | $C_{15}H_{22}O_5$<br>$C_{13}H_{20}N_3O_4$ | octyl gallate<br>isoctyl gallate | X | – | – | 175.0606 | 283.1532 | 98.9738<br>160.0737 |
| 325.0929 | 7.9 | $C_{15}H_{18}O_8$<br>$C_{13}H_{16}N_3O_7$ | 4-*O*-*β*-D-glucosyl-4-hy-droxycinnamate<br>*p*-coumaroyl-D-glucose | X | X | X | 145.0297 | 327.1074 | 147.0445 |
| 355.1029 | 8.6 | $C_{16}H_{20}O_9$<br>$C_{14}H_{18}N_3O_8$ | gentiopicrin | X | – | – | 175.0398 | – | – |
| 229.1443 | 13.7 | $C_{12}H_{22}O_4$<br>$C_{10}H_{20}N_3O_3$ | dodecanoic acid<br>decanedioic acid 1,10-dimethyl ester<br>adipic acid di-*n*-propyl ester | – | – | X | 187.0983 | – | – |
| 231.1590 | 14.2 | $C_{12}H_{24}O_4$<br>$C_{10}H_{22}N_3O_3$ | methyl 9,9-dimethoxynonanoate<br>(2*S*,3*R*)-2,3-dihydroxy-ethyl ester decanoic acid | – | – | X | – | 233.1795 | 98.9746 |
| 229.1440 | 15.0 | $C_{12}H_{22}O_4$<br>$C_{10}H_{20}N_3O_3$ | dodecanoic acid<br>decanedioic acid 1,10-dimethyl ester<br>adipic acid di-*n*-propyl ester | X | – | X | 96.9582 | – | – |
| 263.1283 | 16.0 | $C_{15}H_{20}O_4$ | abscisic acid | X | X | X | 153.0922 | 265.1416 | 135.0809<br>201.1252<br>247.1297 |
| 389.2902 | 18.2 | $C_{21}H_{42}O_6$ | pentadecyl D-glucoside<br>9,10,12,13-tetrahydroxy-henicosanoic acid | X | – | X | – | – | – |
| 327.2538 | 18.8 | $C_{19}H_{36}O_4$ | (2*S*,4*S*)-2,4-dihydroxyhep-tadec-16-enyl acetate | X | X | X | 227.1631 | – | – |
| 329.2333 | 20.9 | $C_{18}H_{34}O_5$<br>$C_{16}H_{32}N_3O_4$ | 9,10,13-trihydroxyoctadec-11-enoic acid | X | X | X | – | - | - |
| 311.2208 | 26.8 | $C_{18}H_{32}O_4$ | (2*S*,4*S*)-2,4-dihydroxyhep-tadec-16-ynyl acetate<br>4-acetoxy-1,2-dihydroxy-heptadec-16-yne | – | – | X | 116.9278<br>209.1148 | – | – |
| 283.2275 | 28.4 | $C_{17}H_{32}O_3$<br>$C_{15}H_{30}N_3O_2$ | 2-methoxy-5*Z*-hexadecenoic acid<br>9-ceto-heptadecilic acid | X | X | X | 145.0618 | 285.2420 | 95.0811<br>135.1108<br>249.2085 |
| 313.2346 | 28.9 | $C_{18}H_{34}O_4$<br>$C_{16}H_{32}N_3O_3$ | (9*Z*)-(7*S*,8*S*)-dihydroxyoctadecenoic acid<br>18-hydroxy-9*R*,10*S*-epoxi-estearic acid | – | – | X | – | – | – |
| 317.2468 | 30.3 | $C_{21}H_{34}O_2$ | (2*E*,5*E*,12*Z*,15*Z*)-1-hydroxy-heneicosa-2,5,12,15-tetraen-4-one | X | – | X | 265.1456 | 319.2633 | 284.2455 |

**Table 4. Continued**

| negative ion polarity | | | | | | | | positive ion polarity | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | variety | | | | | |
| *m/z* exptl | retention time (min) | possibilities in Smart Editor-Molecular formula (upward order of sigma) | possible compounds (for the first formula) | Hass | Lamb−Hass | Rugoro | MS fragments in source | *m/z* exptl | MS fragments in source |
| 315.2506 | 31.1 | $C_{18}H_{36}O_4$<br>$C_{10}H_{38}N_1O_9$ | 9,10-dihydroxystearic acid<br>(9*R*,10*R*)-dihydroxyoctadecanoic acid | X | − | X | − | 317.2475 | 284.2451 |
| 285.2434 | 33.1 | $C_{17}H_{34}O_3$<br>$C_{15}H_{32}N_3O_2$ | 3-hydroxypalmitic acid methyl ester<br>2-methoxyhexadecanoic acid | X | X | − | − | − | − |
| 295.2250 | 33.5 | $C_{18}H_{32}O_3$<br>$C_{16}H_{30}N_3O_2$ | 8-hydroxylinoleic acid<br>(13*S*)-hydroxyocta-decadienoic acid | − | − | X | − | − | − |
| 269.2122 | 35.8 | $C_{16}H_{30}O_3$ | 1,2,4-trihydroxyhep-tadec-16-yne | X | X | X | 116.9290 | 271.2268 | 231.1993 |
| 295.2251 | 36.6 | $C_{18}H_{32}O_3$<br>$C_{16}H_{30}N_3O_2$ | 8-hydroxylinoleic acid<br>(13*S*)-hydroxyoctadec-adienoic acid | − | − | X | − | − | − |
| 237.1850 | 37.3 | $C_{15}H_{26}O_2$<br>$C_{13}H_{24}N_3O_1$ | centarol<br>daucol | X | − | X | − | − | − |
| 313.2747 | 38.2 | $C_{19}H_{38}O_3$ | 1,2,4-trihydroxynonadecane | X | X | X | 225.1853<br>279.1969<br>293.2083 | − | − |
| 275.2381 | 38.9 | $C_{19}H_{32}O_1$<br>$C_{14}H_{32}N_2O_3$ | 2-(1-pentadecenyl)-furan = Avocadofuran | X | X | X | − | 277.2511 | 113.0607<br>267.2297 |
| 277.2535 | 40.4 | $C_{19}H_{34}O_1$ | pentadecylfuran | X | X | X | − | 279.2611 | 95.0847<br>263.2357 |
| 277.2167 | 41.1 | $C_{18}H_{30}O_2$<br>$C_{16}H_{28}N_3O_1$ | linolenic acid<br>4-dodecylresorcinol | X | X | X | 251.2344 | 279.2470 | 95.0850<br>233.2250 |
| 253.2170 | 42.3 | $C_{16}H_{30}O_2$<br>$C_{14}H_{28}N_3O_1$ | palmitoleic acid<br>3-hexadecenoic acid | − | X | − | − | − | − |
| 279.2336 | 43.5 | $C_{18}H_{32}O_2$<br>$C_{16}H_{30}N_3O_1$ | linoleic acid<br>(9*Z*,11*E*)-octadecadienoic acid | X | X | X | − | − | − |
| 319.2638 | 44.1 | $C_{21}H_{36}O_2$ | (2*E*,12*Z*,15*Z*)-1-hydroxy-heneicosa-2,5,12,15-tetraen-4-one | X | X | X | 279.2311 | 321.2798 | 277.2521<br>303.2644 |
| 333.2425 | 44.7 | $C_{21}H_{34}O_3$<br>$C_{19}H_{30}N_3O_1$ | 4-tetradecoxybenzoic acid<br>tetradecyl 4-hydroxybenzoate<br>tridecyl 3-methoxybenzoate | − | X | − | 251.2367<br>279.2313 | 335.2581 | 277.2561<br>295.2639 |
| 255.2322 | 46.1 | $C_{16}H_{32}O_2$<br>$C_{14}H_{30}N_3O_1$ | palmitic acid<br>myristyl acetate | X | X | X | − | 257.2374 | 219.2111 |
| 281.2491 | 47.0 | $C_{18}H_{34}O_2$<br>$C_{16}H_{32}N_3O_1$ | oleic acid<br>elaidic acid | X | X | X | − | − | − |
| 283.2635 | 49.9 | $C_{18}H_{36}O_2$<br>$C_{16}H_{34}N_3O_1$ | stearic acid<br>ethyl palmitate<br>isooctadecanoic acid | − | X | X | − | − | − |

[a] In some cases, in positive polarity, the in-source fragments were most intense than $[M + H]^+$; these are underlined. Error (ppm) and mSigma values were lower than 4.7 and 25.1 in every case. X means that the compound was present in the avocado sample. − means that the compound was not present in the avocado sample.

**Repeatability Study.** Repeatability was studied by performing a series of separations using the optimized method on one of the samples the same day (intraday precision, $n = 12$) and on three consecutive days (interday precision, $n = 36$). The relative standard deviations (RSDs) of peak areas/retention time were determined by assaying seven of the compounds present in the extracts (protocatechuic acid, chlorogenic acid, epicatechin, ferulic acid, kaempferide, and compounds with $m/z$ 315.2525 and 295.2264).

The intraday repeatabilities on the total peak area/retention time (expressed as RSD) were between 1.7 and 3.1%, whereas the interday repeatabilities on total peak area/retention time were found in the range from 2.5 to 4.6%.

In summary, the separation by an effective HPLC profiling method with online detection by DAD and ESI-TOF-MS was successfully applied to the analysis of the secondary metabolites (phenolic compounds and some other components) extracted from avocado samples. Our LC-DAD/ESI-TOF MS method can determine more than 40 analytes in each run, 17 of which were unequivocally identified by using standards; for the rest, we achieved a tentative identification considering the information provided by the powerful TOF, the chemical information that we can obtain from the chromatographic separation (polarity and size of the compounds), and the information previously published.

Significant differences were found when the analyses of the three varieties used in this study were compared. In our laboratory there are already some studies ongoing using more varieties of avocado fruit, making a proper in-depth comparison by using statistical tools looking for potential varietal markers.

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## REFERENCES


(1) Stalikas, C. D. Extraction, separation and detection methods for phenolic acids and flavonoids. *J. Sep. Sci.* **2007**, *30*, 3268–3295.

(2) Naczk, M.; Shahidi, F. Phenolics in cereals, fruits and vegetables: occurrence, extraction and analysis. *J. Pharm. Biomed.* **2006**, *41*, 1523–1542.

(3) Hounsome, N.; Hounsome, B.; Tomos, D.; Edwards-Jones, G. Plant metabolites and nutritional quality of vegetables. *J. Food Sci.* **2008**, *73*, 48–65.

(4) Crozier, A.; Yokota, T.; Jaganath, I. B.; Marks, S. C.; Saltmarsh, M.; Clifford, M. N. Secondary metabolites in fruits, vegetables, beverages and other plant-based dietary components. In *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet*; Crozier, A., Clifford, M. N., Ashihara, H., Eds.; Blackwell Publishing: Oxford, U.K., 2006; pp 208−302.

(5) Puupponen-Pimiä, R.; Nohynek, L.; Hartmann-Schmidlin, S.; Kähkönen, M.; Heinonen, M.; Määttä-Riihinen, K.; Oksman-Caldentey, K. M. Berry phenolics selectively inhibit the growth of intestinal pathogens. *J. Appl. Micorbiol.* **2005**, *98*, 991–1000.

(6) Rotelli, A. E.; Guardia, T.; Juárez, A. O.; de la Rocha, N. E.; Pelzer, L. E. Comparative study of flavonoids in experimental models of inflammation. *Pharmacol. Res.* **2003**, *48*, 601–606.

(7) Mamani-Matsuda, M.; Kauss, T.; Al-Kharrat, A.; Rambert, J.; Fawaz, F.; Thiolat, D.; Moynet, D.; Coves, S.; Malvy, D.; Mossalayi, M. D. Therapeutic and preventive properties of quercetin in experimental arthritis correlate with decreased macrophage inflammatory mediators. *Biochem. Pharmacol.* **2006**, *72*, 1304–1310.

(8) Gasiorowski, K.; Szyba, K.; Brokos, B.; Kolaczyńska, B.; Jankowiak-Wlodarczyk, M. M.; Oszmiański, J. Antimutagenic activity of anthocyanins isolated from *Aronia melanocarpa* fruits. *Cancer Lett.* **1997**, *119*, 37–46.

(9) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S–2085S.

(10) Ashton, O. B. O.; Wong, M.; McGhie, T. K.; Vather, R.; Wang, Y.; Requejo-Jackman, C.; Ramankutty, P.; Woolf, A. B. Pigments in avocado tissue and oil. *J. Agric. Food Chem.* **2006**, *54*, 10151–10158.

(11) Ding, H.; Chin, Y.-W.; Kinghorn, A. D.; D'Ambrosio, S. M. Chemopreventive characteristics of avocado fruit. *Semin. Cancer Biol.* **2007**, *17*, 386–394.

(12) Henrotin, Y. Avocado/soybean unsaponifiable (ASU) to treat osteoarthritis: a clarification. *Osteoarthr. Cartilage* **2008**, *16*, 1118–1119.

(13) Borrone, J. W.; Brown, J. S.; Tondo, C. L.; Mauro-Herrera, M.; Kuhn, D. N.; Violi, H. A.; Sautter, R. T.; Schnell, R. J. An EST-SSR-based linkage map for *Persea americana* Mill. (avocado). *Tree Genetics Genomes* **2009**, *5*, 553–560.

(14) Harnly, J. M.; Bhagwat, S.; Lin, L.-Z. Profiling methods for the determination of phenolic compounds in foods and dietary supplements. *Anal. Bioanal. Chem.* **2007**, *389*, 47–61.

(15) Lin, L.-Z.; Harnly, J. M. A screening method for the identification of glycosylated flavonoids and other phenolic compounds using a standard analytical approach for all plant materials. *J. Agric. Food Chem.* **2007**, *55*, 1084–1096.

(16) Hurtado-Fernández, E.; Gómez-Romero, M.; Carrasco-Pancorbo, A.; Fernández-Gutiérrez, A. Application and potential of capillary electroseparation methods to determine antioxidant phenolic compounds from plant food material. *J. Pharm. Biomed.* **2010**, *53*, 1130–1160.

(17) Torres, A. M.; Mau-Lastovicka, T.; Rezaaiyan, R. Total phenolics and high-performance liquid chromatography of phenolic acids of avocado. *J. Agric. Food Chem.* **1987**, *35*, 921–925.

(18) Mattila, P.; Hellström, J. Phenolic acids in potatoes, vegetables and some of their products. *J. Food Compos. Anal.* **2007**, *20*, 152–160.

(19) de Pascual-Teresa, S.; Santos-Buelga, C.; Rivas-Gonzalo, J. C. Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. *J. Agric. Food Chem.* **2000**, *48*, 5331–5337.

(20) Arts, I. C. W.; van de Putte, B.; Hollman, P. C. H. Catechin contents of foods commonly consumed in the Netherlands. 1. Fruits, vegetables, staple foods, and processed foods. *J. Agric. Food Chem.* **2000**, *48*, 1746–1751.

(21) Harnly, J. M.; Doherty, R. F.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Bhagwat, S.; Gebhardt, S. Flavonoid content of U.S. fruits, vegetables and nuts. *J. Agric. Food Chem.* **2006**, *54*, 9966–9977.

(22) Miean, K. H.; Mohamed, S. Flavonoid (myricetin, quercetin, kaempferol, luteolin and apigenin) content of edible tropical plants. *J. Agric. Food Chem.* **2001**, *49*, 3106–3112.

(23) Fukuji, T. S.; Tonin, F. G.; Tavares, M. F. M. Optimization of a method for determination of phenolic acids in exotic fruits by capillary electrophoresis. *J. Pharm. Biomed.* **2010**, *51*, 430–438.

(24) Mertz, C.; Gancel, A.-L.; Gunata, Z.; Alter, P.; Dhuique-Mayer, C.; Vaillant, F.; Perez, A. M.; Ruales, J.; Brat, P. Phenolic compounds, carotenoids and antioxidant activity of three tropical fruits. *J. Food Compos. Anal.* **2009**, *22*, 381–387.

(25) Barreto, J. C.; Trevisan, M. T. S.; Hull, W. E.; Erben, G.; de Brito, E. S.; Pfundstein, B.; Würtele, G.; Spiegelhalder, B.; Owen, R. W. Characterization and quantitation of polyphenolic compounds in bark, leaves and peel of mango (*Mangifera indica* L.). *J. Agric. Food Chem.* **2008**, *56*, 5599–5610.

(26) Simirgiotis, M. J.; Caligari, P. D. S.; Schmeda-Hirschmann, G. Identification of phenolic compounds from the fruits of the mountain papaya *Vasconcellea pubescens* A. DC. grown in Chile by liquid chromatography−UV detection−mass spectrometry. *Food Chem.* **2009**, *115*, 775–784.

(27) Määttä-Riihinen, K. R.; Kamal-Eldin, A.; Riitta Törrönen, A. Identification and quantification of phenolic compounds in berries of *Fragaria* and *Rubus* species (family Rosaceae). *J. Agric. Food Chem.* **2004**, *52*, 6178–6187.

(28) Naczk, M.; Shahidi, F. Extraction and analysis of phenolics in food. *J. Chromatogr., A* **2004**, *1054*, 95–111.

(29) Antolovich, M.; Prenzler, P.; Robards, K.; Ryan, D. Sample preparation in the determination of phenolic compounds in fruits. *Analyst* **2000**, *125*, 989–1009.