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Manuscript Draft

Manuscript Number:

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Article Type: Research Paper

Keywords: By-products; phenolic compounds; grape; rapid resolution liquid chromatography (RRLC); mass spectrometric (MS)

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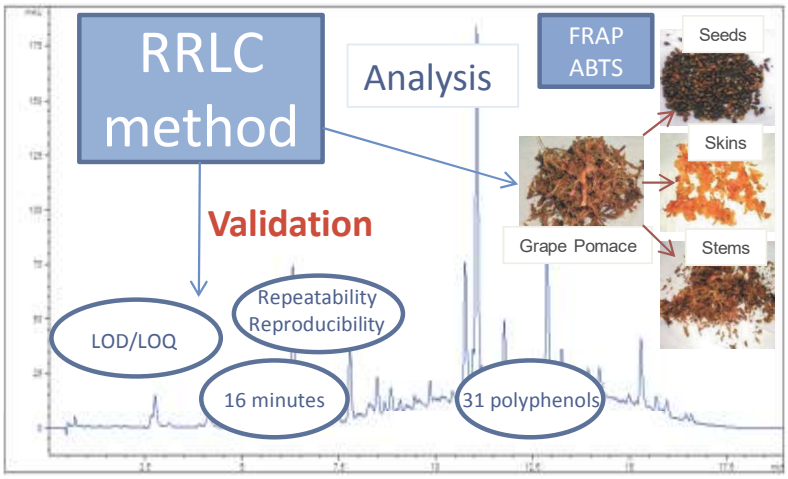
Abstract: The development and validation of a rapid method of RRLC has been carried out to determine the phenolic composition of winemaking by-products (pomaces, seeds, skins and stems). Thirty-one phenolic compounds belonging to three groups (flavanols, flavonols and phenolic acids) have been identified by use of standards and mass spectrometric detection, and quantified by using the corresponding external standard calibration plot, in a 16-minute run. The validation was realized calculating the repeatability, the reproducibility and the limits of detection (LOD) and quantification (LOQ), from standards solutions. The limits of detection and quantification were in the range of 0.16-1.09 and 0.52-3.63 mg/L, respectively, and good repeatability (R.S.D. values < 1.5%) and reproducibility (R.S.D. values < 5.5%) were found. Results confirmed that the method is effective and suitable for determination of phenolic compounds in winemaking by-products. Seeds, skins, stems and pomaces exhibited a different qualitative and quantitative phenolic profile and different antioxidant activities.

Highlights

1. A rapid RRLC method has been validated to determine the phenolic composition of winemaking by-products.
2. Pomaces, seeds, skins and stems have been analyzed by RRLC and MS.
3. Thirty-one phenolic compounds have been identified and quantified in samples in a 16-minute run.
4. Samples exhibited different phenolic profile and antioxidant activity.

*Graphical Abstract (for review)



Detailed phenolic composition of white grape by-products by RRLC/MS and measurement of the antioxidant activity.

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Abstract

The development and validation of a rapid method of RRLC has been carried out to determine the phenolic composition of winemaking by-products (pomaces, seeds, skins and stems). Thirty-one phenolic compounds belonging to three groups (flavanols, flavonols and phenolic acids) have been identified by use of standards and mass spectrometric detection, and quantified by using the corresponding external standard calibration plot, in a 16-minute run. The validation was realized calculating the repeatability, the reproducibility and the limits of detection (LOD) and quantification (LOQ), from standards solutions. The limits of detection and quantification were in the range of 0.16-1.09 and 0.52-3.63 mg/L, respectively, and good repeatability (R.S.D. values < 1.5%) and reproducibility (R.S.D. values < 5.5%) were found. Results confirmed that the method is effective and suitable for determination of phenolic compounds in winemaking by-products. Seeds, skins, stems and pomaces exhibited a different qualitative and quantitative phenolic profile and different antioxidant activities.

Keywords

By-products; phenolic compounds; grape; rapid resolution liquid chromatography (RRLC); mass spectrometric (MS).

INTRODUCTION

Phenolic compounds have been widely studied for decades because of their beneficial properties on the health and their influence on the organoleptic characteristics of the food. Grape pomace, consisting of seeds, skins and stems, is a winemaking by-product recognized as a rich source in phenolic compounds with interest by their potential natural antioxidant [1], anti-inflammatory [2] and antimicrobial activities [3], which have been related with the prevention of important chronic pathologies such as cardiovascular disorders [4], neurodegenerative decline [5] or cancer [6].

Winemaking generates a high amount of by-products that cause environmental and economic problems, which could be minimized by the exploitation and valorisation of those products, such as their use in pharmaceutical and food industries.

Seeds, skins and stems present different qualitative and quantitative composition in phenolic compounds. Seeds and stems are rich in flavanols whereas skins also present flavonols, and it is well known that different phenolic compounds may show different biological and antioxidant properties [7]. Several *in vitro* methods had been employed to measure the antioxidant activity, such as ABTS and DPPH assays, and ferric reducing antioxidant power (FRAP), based on an electron transfer mechanism and reduction of a coloured oxidant, and others based on a hydrogen atom transfer mechanism, such as oxygen radical absorbance capacity (ORAC), in which antioxidants and substrate compete for thermally generated peroxyl radicals [8,9].

Different techniques have been used for the separation of phenolic compounds, such as high speed counter current chromatography (HSCCC), supercritical fluid chromatography (SFC), capillary electrophoresis (CE) and especially high performance liquid chromatography (HPLC), the most commonly used for the separation and analysis of these compounds in grape, wine and related products [10]. However, these

methods either require longer analysis time or consume relatively large amounts of organic solvents used as mobile phase. Considering the complexity of the grape pomace, with a diversity of phenolic compounds from different groups, it is very difficult to achieve good separations with a single chromatographic run. Several authors have analysed polyphenols in seeds, skins and stems using different chromatographic conditions with good results but usually requiring long retention times [1,11-13]. Rapid resolution liquid chromatography (RRLC) is a technique of liquid chromatography in which small particles are packed into short columns run with small particle size and diameter. The advantages of RRLC are higher resolution and sensitivity, and shorter retention times than HPLC. However, as far as we know, this technique has not been used for the analysis of phenolics in grape pomace. Liquid-liquid extraction has been widely used in sample preparation for further analysis of phenolic compounds. In this regard, it is important to stress that the choice of the extraction solvents must be made as a function of the type of sample to be analysed and the information required [14]. Thus, several extraction solvents (ethanol, methanol, ethyl acetate, and sulphured water) have been used for the analysis of phenolic compounds in grape seeds [14-16], grape skin and seeds [17], or grape pomace [18].

The aim of this work was to determine the phenolic composition of white grape winemaking by-products (pomaces, seeds, skins and stems) by the development and validation of a rapid and effective RRLC method using MS as a detection technique for compound identification and/or confirmation. In addition, the antioxidant activity of the by-product extracts was measured by ABTS and FRAPS assays, and correlations with the phenolic composition were established.

MATERIALS AND METHODS

Samples and Reagents

Grape pomace of the variety Zalema, D.O. "Condado de Huelva" (Spain) from the 2011 harvest collected after winemaking was supplied by "Vinícola del Condado" winery (Bollullos Par del Condado, Spain). Seeds, skins and stems were manually separated from the grape pomace samples and all samples were further freeze-dried.

Hydrochloric acid, formic acid, HPLC-grade acetonitrile, methanol, ethanol, glycine, Folin reagent, and iron trichloride ($FeCl_3 \cdot 6H_2O$) were obtained from Panreac (Barcelona, Spain). ABTS (2,2-azino-bis-(3-ethylbenzothiazolne-6-sulfonic acid) diammonium salt) and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) were purchased from Fluka (Madrid, Spain).

Gallic acid, protocatechuic acid, (+)-catechin (C), (-)-epicatechin (EC), quercetin, kaempferol, ferulic acid, caffeic acid, *p*-coumaric acid, sodium carbonate, potassium persulphate, potassium metabisulphite, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (Madrid, Spain). Quercetin 3-*O*-glucoside and kaempferol 3-*O*-glucoside were obtained from Extrasynthese (Lyon, France). Procyanidin dimers B1, B2, B3 and B4 and trimer C1 were isolated in the laboratory by semi-preparative HPLC [19].

Sample preparation

The ability of different solvents to extract the polyphenols in the by-products samples (pomace, seeds, skins and stems) was investigated. For this, analyses were carried out after extraction with four different solvents: 70% ethanol, 40% ethanol, 75% methanol and 1% potassium metabisulphite, in water. The by-product sample (5 g) was homogenized in 25 mL of the solvent, kept under shaking for 1 h in an incubating mini shaker (VWR International, Barcelona. Spain), and further centrifuged at 4190*g* for 15 min; the

supernatant was collected and the residue submitted to the same process twice, and the supernatants combined. The extracts thus obtained were used for determination of total phenols content by spectrophotometry, and the average recoveries were selected as responses of interest.

After selection of the solvent, the extracts obtained was used for determination of the antioxidant activity by FRAP and ABTS assays. Furthermore, the extracts (2 mL) were concentrated to dryness and further re-dissolved in 1 mL of 0.1% formic acid to be analysed by RRLC after filtration through a hydrophilic PVDF Millex-HV 0.45 μm syringe filter (Millipore, Bedford, MA, USA).

Chromatography

Analyses were carried out in an Agilent 1260 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode-array detector, which was set to scan from 200 to 770 nm, and a C18 Poroshell 120 column (2.7 μm, 5 cm x 4.6 mm) using an injection volume of 15 μL.

The solvents were 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) at the following gradient: 0-5 min, 5% B linear; 5-20 min 50% B linear; 20-25 min, washing and re-equilibration of the column. The flow-rate was 1.5 mL/min and the temperature of the column was set at 25 ºC. Detection was also performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyser, which was connected to the HPLC equipment via the DAD cell outlet, as described by Jara-Palacios et al. [9]. Phenolic compounds were identified by their retention time, UV-vis spectra and mass spectra, as well as by comparison with our data library and standards when available.

Analytical Quality Control

The quantification of the phenolic compounds was carried out by external calibration from the areas of the chromatographic peaks obtained by UV detection at the following wavelengths: 280 nm for benzoic acids and flavanols, 320 nm for cinnamic acid derivatives and 370 nm for flavonols. The stock solutions of phenolic standards were prepared in acetonitrile at a concentration of 100 mg/L. The corresponding calibration curves were made up of six dilutions of the stock solutions in 0.1% formic acid for the following polyphenols: catechin, epicatechin, gallic acid, protocatechic acid, caffeic acid, ferulic acid, *p*-coumaric acid, quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, quercetin and kaempferol. Procyanidins were quantified with the calibration curve of catechin. Caftaric, fertaric and coutaric acids were quantified using the calibration curves of caffeic, ferulic and *p*-coumaric acids, respectively. Quercetin and isorhamnetin derivatives were quantified as quercetin 3-*O*-glucoside and kaempferol derivates as kaempferol-3-*O*-glucoside.

The limits of detection (LOD) and quantification (LOQ) were calculated from the calibration curves, using the Microcal Origin *ver. 3.5sofware* (OriginLab Corporation, Northampton, MA, USA). The LOD were calculated as three times the relative standard deviation of the analytical blank values calculated from the calibration curve. The LOQ were calculated as ten times the relative standard deviation of the analytical blank values calculated from the calibration curve.

The within-laboratory repeatability (within-day precision) was developed according to UNE 82009 standard [20]. It was ascertained by analysing the phenolic content in a standard solution, under the same analytical conditions, six times within the same day. Within-laboratory reproducibility (day-to-day precision) was assessed by analysing in

duplicate a standard solution over a period of 1 month, the control sample being kept at –20 ºC between the analyses.

Three replicates from each sample to quantify each compound were analysed and all the samples and standards were injected three times to obtain the averages.

Total phenolic content

Total phenolic content was determined using the Folin-Ciocalteau assay [21]. Briefly, 0.25 mL of extract (pomace, seeds, skins or stems), 1.25 mL of Folin-Ciocalteu reagent, and 3.75 mL of a solution of sodium carbonate (20%) were mixed and distilled water was added to make up a total volume of 25 mL. The solution was homogenized and left to stand for 120 min for the reaction to take place. Then, the absorbance was read at 765 nm with a Hewlett-Packard UV-vis HP8453 spectrophotometer (Palo Alto, CA, USA). Gallic acid was employed as a calibration standard and results were expressed as gallic acid equivalents (mg GAE/g of dry matter).

FRAP assay

Ferric reducing ability was evaluated according to Benzie and Strain [22] with some modifications. The FRAP reagent contained 10 mM of TPTZ solution in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$, and acetate buffer (300 mM, pH 3.6) (1:1:10, v/v/v). A 100-µL of extract (pomace, seed, skin or stem) was added to 3 mL of the FRAP reagent and the absorbance was measured at 593 nm after incubation at room temperature for 6 min, using the FRAP reagent as a blank. Different dilutions of each extract were assayed and the results were obtained by interpolating the absorbance on a calibration curve obtained with Trolox (30-1,000 µM). Three independent experiments in triplicate were performed for each of the assayed extracts and the results were expressed as Trolox-equivalent antioxidant capacity (TEAC), here considered as the mmols of Trolox with the same antioxidant capacity as 100 g of the studied extract.

ABTS/persulphate assay

The $ABTS^{\bullet+}$ radical was produced by the oxidation of 7 mM ABTS with potassium persulphate (2.45 mM) in water [23]. The mixture was allowed to stand in the dark at room temperature for 16 h before use, and then the $ABTS^{\bullet+}$ solution was diluted with phosphate buffered saline (PBS) at pH 7.4 to give an absorbance of 0.7±0.02 at 734 nm. The extracts (50 μL) of pomace, seed, skin or stem were mixed with 2 mL of the $ABTS^{\bullet+}$ diluted solution, vortexed for 10 s, and the absorbance measured at 734 nm after 4 min of reaction at 30 ºC.

Different dilutions of each extract were assayed and the results were obtained by interpolating the absorbance on a calibration curve obtained with Trolox (30-1,000 μM). Three independent experiments were performed in triplicate for each of the assayed extracts and the results were expressed as Trolox-equivalent antioxidant capacity (TEAC; mmols of Trolox with the same antioxidant capacity as 100 g of the studied extract).

Statistical analysis

For the statistical treatment of the data the Statistica v.8.0 software [24] was used. One-way analysis of variance (ANOVA) was employed to establish if phenolic composition differed significantly between: a) the extracts obtained with different solvents and b) the different winemaking by-products (seeds, skins, stems, pomaces). In addition, correlations between the contents of total phenolics determined by RRLC and the antioxidant activity were studied. In all cases, statistically significant level was considered at $p<0.05$. Pattern recognition (PR) techniques, like stepwise linear discriminant analysis (SLDA), were applied on experimental standardized data to distinguish between different types of by-products.

RESULTS AND DISCUSSION

Different assays were carried out to optimize the chromatographic conditions in order to obtain suitable separation of the phenolic compounds in the extracts. For this, a mixture of standards and a grape pomace extract were used. Figure 1 shows the chromatogram of the standard mixture in the optimized conditions, detailed in section 2.3. The developed method allows the separation of up to thirty-one phenolic compounds in the winery by-products in a 16-minute run, belonging to three different groups: phenolic acids i.e. (a) benzoic acids (gallic and protocatechuic acids); (b) hydroxycinnamoyl derivatives (caffeic, caftaric, fertaric, and *cis*- and *trans*-coutaric acids); flavanols (catechin, epicatechin, procyanidins B1, B2, B3, B4, B7 and B2 3-*O*-gallate, two trimers, two tetramers and one galloyled procyanidin), and flavonols (quercetin and kaempferol aglycones, and four quercetin, three kaempferol and two isorhamnetin derivatives).

Analytical characteristics

The calibration curves were constructed with six levels of concentration in triplicate. All the curves showed good linearity (r^2> 0.9975) in the range of concentrations studied (Table 1). The lowest LOD and LOQ corresponded to epicatechin (0.16 mg/L and 0.52 mg/L, respectively) and the highest ones to quercetin-3-*O*-glucoside (1.09 mg/L and 3.63 mg/L, respectively).

The repeatability and reproducibility were evaluated by the relative standard deviation for the retention times and peaks areas of the standards solution (Table 2). Concerning the repeatability, the RSD values were under 1.46 %. The highest values corresponded to quercetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside, 0.17% (for retention time), and kaempferol-3-*O*-glucoside, 1.46% (for peaks area). The highest RSD observed in the reproducibility corresponded to gallic acid (0.26%) and catechin (5.52%), for

retention times and peak area, respectively. Nonetheless, most of the RSD values obtained were below 5.52%, which confirmed the high reproducibility of the method.

Analysis of phenolic compounds in the by-products

Four different extraction solvents were tested for the extraction of the phenolic compounds from the winery by-products and the average recoveries corresponding to the total phenolic content were selected as responses of interest. The results are shown in Fig. 2. Significant differences ($p<0.05$) in the extraction of phenolic compounds were found depending on the solvent. The highest extraction efficiency was achieved with 75% methanol in all by-products samples, followed by 70% ethanol, 40% ethanol and 1% potassium metabisulphite. The average recoveries obtained with 75% methanol were some three-fold higher to those obtained with potassium metabisulphite in seeds and skins, and some two-fold higher in stems and pomaces. As for aqueous ethanol, the average recoveries decreased when the percentage of alcohol in the solvent was lower, although this effect was not statistically significant at $p>0.05$. Considering these results, 75% methanol was selected as solvent to obtain the extracts from by-products samples.

Identification of individual phenolic compounds

A total of thirty-one different compounds were identified and quantified in the different by-products (Table 3). Compounds were identified according to their mass characteristics and also chromatographic behaviour and absorption spectra in comparison with available standards or library data. Flavanols monomers (i.e., catechin and epicatechin) exhibited their deprotonated molecular ion $[M-H]^-$ at *m*/*z* 289. Other flavanols detected were procyanidin dimers (B1, B2, B3, B4 and B7; pseudomolecular ion $[M-H]^-$ at *m*/*z* 577), trimers ($[M-H]^-$ at *m*/*z* 865) and tetramers ($[M-H]^-$ at *m/z* 1153). Also, two compounds were identified as galloyled procyanidin dimers ($[M-H]^-$

at *m*/*z* 729), one of which was confirmed to be procyanidin B2-3-*O*-gallate by comparison with a standard available in the laboratory.

Eleven compounds were associated to flavonols based on their characteristics absorption spectra showing maximum wavelengths around 350-370 nm. Quercetin ([M-H]$^-$ at *m*/*z* 301), kaempferol ([M-H]$^-$ at *m*/*z* 285) aglycones and some glycoside derivatives from them and isorhamnetin (product ion corresponding to the aglycone at *m*/*z* 315) were detected. Glycosides were assigned based on the characteristics losses of fragments, i.e., 162 mu (glucosides), 176 mu (glucuronides), 308 mu (rutinosides) or 132 (pentosides). The identity of some of them was further established by comparison with available standards.

Two hydroxybenzoic acids, i.e., gallic acid (pseudomolecular ion [M-H]$^-$ at *m*/*z* 169 releasing a product ion at *m/z* 125 by loss of CO_2, -44 mu) and protocatechuic acid ([M-H]$^-$ at *m*/*z* 153) and a hydroxycinnamic acid (caffeic acid; [M-H]$^-$ at *m*/*z* 179) were also identified, as well as four hydroxycinnamoyl-tartaric esters: caftaric ([M-H]$^-$ at *m/z* 311), fertaric ([M-H]$^-$ at *m/z* 325), *cis*-coutaric ([M-H]$^-$ at *m/z* 295) and *trans*-coutaric acids ([M-H]$^-$ at *m/z* 295), whose identity was established by comparison with data in the literature [11,25,26].

Quantification of phenolic compounds in the winery by-products

Different quantitative phenolic profiles (Table 3) were found in the distinct by-products (seeds, skins, stems and pomace), which showed significant differences ($p<0.05$) in their contents of flavanols, flavonols and phenolic acid derivatives (Fig. 3). In general, flavanols were the most abundant phenolics, with concentrations ranging between 121 and 613 mg/100 g dry matter, followed by flavonols (8-146 mg/100 g) and phenolic acids (9-27 mg/100 g). The highest amounts of flavanols were found in seeds (613 mg/100 g), followed by stems (348 mg/100 g) and pomace (282 mg/100 g), while skins

297 presented the lowest concentration (122 mg/100 g). In contrast, flavonols were most
298 abundant in skins and pomace (146 and 144 mg/100 g, respectively) with no significant
299 differences between them, whereas seeds were poor in these compounds skins (8
300 mg/100 g), as also reported by other authors [11]. Phenolic acid derivatives were
301 minority compounds in the four by-products (Fig. 3).

302 Considering individual compounds, procyanidins B1 and B2 3-*O*-gallate and catechin
303 were the predominant flavanols in all by-products. The highest amounts of procyanidin
304 B2 3-*O*-gallate and catechin were found in seeds (146 and 65 mg/100 g, respectively)
305 and procyanidin B1 was highest in stems and seeds (89 and 85 mg/100 g, respectively).
306 Procyanidin B2, B3, B4 and B7 were also found in all by-products, being B4 the
307 predominant non-galloyled dimer after B1. A not identified galloyled procyanidin was
308 also found in relatively high amount in seeds (60 mg/100 g) while it was in lower levels
309 in the other extracts (between 4 and 26 mg/100 g). Epicatechin, which was described as
310 an important flavanol in by-products from grape and wine [13], showed very different
311 concentrations among by-products ranging between 5 and 43 mg/100 g, being more
312 abundant in seeds than in skins. As for other flavanols, procyanidin trimers and
313 tetramers also showed relevant contribution to the levels of total flavanols in seeds,
314 stems, pomace and skins (114, 54, 48 and 20 mg/100 g).

315 The main flavonols in the by-products were quercetin glycosides, with quercetin 3-*O*-
316 glucoside and quercetin 3-*O*-glucuronide, accounting for 32-42% and for 35-37% of
317 total flavonol content, respectively. As expected, both compounds were more abundant
318 in pomace and skins (51-58 mg/100 g) than in stem (25-27 mg/100 g) and seed extracts
319 (around 3 mg/100 g). These compounds have been also identified as the most abundant
320 flavonols in other grape varieties grown in warm climate Spanish regions, such as
321 Airén, Chardonnay, Listán Huelva, Pedro Ximénez or Verdejo [27]. Other flavonol

glycosides were also found in the by-products but in lower concentrations than quercetin glycosides, being among them kaempferol 3-*O*-glucoside the most abundant in skins and pomaces (19 and 15 mg/100 g, respectively). Isorhamnetin 3-*O*-glucoside was also found in the extracts in low concentrations as reported by other authors [27,28]. These results are in accordance with Castillo-Muñoz et al. [27] that reported quercetin glycosides as the dominant flavanols in white grapes, followed by kaempferol glycosides, considered as the second in importance, and isorhamnetin glycosides that occurred as very minor flavonols. In this study, quercetin and kaempferol aglycones, which were not reported by other authors [27,29], were also detected in low concentrations in the extracts of skins, stems and pomaces.

Gallic acid was the most abundant non-flavonoid phenolic compound in the samples, representing between 36% (skins) and 74% (pomace) of the total contents of phenolic acids and derivatives. Caftaric acid was the most abundant cinnamoyl derivative in all by-products, showing higher concentrations in pomaces and stems (10 and 7 mg/100 g, respectively) than in seeds and skins (around 2 mg/100 g). Rodriguez-Montealegre et al. [28] described *trans*-caftaric as the main acid in white grape skins although they did not identify it in the seeds.

Antioxidant activity in the studied by-products

The antioxidant activity of seeds, skins, stems and pomaces was measured by ABTS and FRAP assays (Table 4). Seed extracts presented the greatest antioxidant capacity (97 mmol TE/100g) in the ABTS assay, followed by pomace, stem, and skin extracts (70, 52 and 42 mmol TE/100 g, respectively), with significant differences among them ($p<0.05$). Similar results were obtained in the FRAP assay for seed and pomace (39 and 35 mmol TE/100 g, respectively) and for stem and skin extracts (22 and 20 mmol TE/100 g, respectively). These results were in agreement with the total phenolic

contents determined by RRLC, which was significantly correlated with ABTS (r^2 = 0.89, $p<0.05$) and FRAP (r^2 = 0.70, $p<0.05$) values, as showed by regression analysis. Different authors also reported significant correlation between antioxidant activity and the total phenolic content in winemaking by-products from different grape varieties [12,16,30].

SLDA analysis

To ascertain whether it was possible to discriminate between pomace, seeds, skins and stems as a function of the phenolic contents, one stepwise linear discriminant analysis (SLDA) was carried out. Eight variables were found significant ($p<0.05$): trimer 2, tetramer 1, *cis*-coutaric acid, quercetin 3-*O*-glucuronide, protocatechuic acid, caftaric acid, kaempferol and procyanidin B2, indicated in descending order of discriminating power. Two classification functions were obtained, which yielded a good separation (100% correct classification) among samples (Fig. 4). The discriminant function 1 was mainly related to trimer 2, procyanidin B2 and protocatechuic acid (with positive sign), and kaempferol (negative sign), whereas the discriminant function 2 was mainly linked to quercetin 3-*O*-glucuronide, caftaric acid and *cis*-coutaric acid (positive sign), and tetramer 1 (negative sign).

CONCLUSIONS

A chromatographic method for the rapid analysis of phenolic compounds in extracts of winemaking by-products has been described, whose applicability is demonstrated by validation criteria considering the linearity, repeatability and reproducibility. Analysis of real samples of by-products has been further carried out, which constitutes, in our knowledge, the first investigation of the simultaneous identification and quantification by RRLC/MS of phenolic compounds belonging to different phenolic groups in

different winery by-products (pomace, seeds, skins and stems). Thirty-one phenolic compounds were identified in the different samples belonging to the groups of flavanols, flavonols and phenolic acid derivatives, showing quantitative differences among the distinct by-products from the white grape Zalema, a *Vitis vinifera* variety used for wine production in the D.O. "Condado de Huelva" in south Spain. Eight phenolic compounds (a procyanidin trimer, a procyanidin tetramer, *cis*-coutaric acid, quercetin 3-*O*-glucuronide, protocatechuic acid, caftaric acid, kaempferol, and procyanidin B2) allowed classifying correctly 100% of the by-product samples. The antioxidant activity of the different by-product samples was also determined by the FRAP and ABTS assays and a correlation between it and the phenolic composition was established.

Acknowledgements

The authors acknowledge the collaboration of "Consejo Regulador D.O. Condado de Huelva". M.J.J.P. holds a predoctoral research grant (FPU) from the Spanish Ministry of Education. The GIP-USAL is financially supported by the Spanish Government through the project CSD2007-00063 (Fun-*c*-Food, Consolider-Ingenio 2010 Programme).

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FIGURE CAPTIONS

Figure 1. RRLC chromatograms recorded at 280, 320 and 370 nm of a mixture of standards in the optimized chromatography conditions. Peaks: 1, gallic acid; 2, protocatechuic acid; 3, catechin; 4, epicatechin; 5, caffeic acid; 6, *p*-coumaric acid; 7, ferulic acid; 8, quercetin 3-*O*-glucoside; 9, kaempferol 3-*O*-glucoside; 10, quercetin; 11, kaempferol.

Figure 2. Average recoveries corresponding to the total phenolic content after extraction of seeds, skins, stems and pomace using four different extraction solvents. Different letters in the same by-product indicate significant differences by ANOVA test ($p<0.05$).

Figure 3. Concentration of flavanols, flavonols and phenolic acids in seeds, skins, stems and pomace of *Vitis vinifera* cv. Zalema. Different letters in the same phenolic group indicate significant differences by ANOVA test ($p<0.05$).

Figure 4. Scatterplot of the by-products samples in the plane defined by the canonical function when phenolic composition is considered for discrimination.

Table 1. Analytical parameters of calibration curves of standards solutions.

Compound	Wavelength (nm)	Intercept ± SD	Slope ± SD	Correlation coefficient (r^2)	Linear range (mg/L)	LOD (mg/L)	LOQ (mg/L)
Catechin	280	-0.368 ± 0.77	12.016 ± 0.00	1.0000	0.4-500	0.192	0.641
Epicatechin	280	-1.506 ± 0.61	11.838 ± 0.00	1.0000	0.4-500	0.155	0.518
Quercetin	370	9.299 ± 11.07	95.705 ± 0.25	0.9998	1.5-100	0.347	1.156
Kaempferol	370	3.841 ± 9.38	79.675 ± 0.22	0.9998	1.5-100	0.353	1.778
Q-3-*O*-glucoside	370	-4.395 ± 7.62	21.026 ± 0.08	0.9994	0.9-250	1.088	3.628
K-3-*O*-glucoside	370	-4.0479 ± 3.89	20.222 ± 0.20	0.9975	0.2-50	0.578	1.927
Gallic acid	280	-7.100 ± 2.88	35.214 ± 0.05	0.9987	1.5-150	0.245	0.818
Protocatechuic acid	280	-4.499 ± 1.22	23.118 ± 0.12	0.9998	0.2-25	0.158	0.527
Caffeic acid	320	-34.080 ± 9.83	66.639 ± 0.28	0.9995	0.2-100	0.442	1.475
Ferulic acid	320	-7.028 ± 4.66	84.166 ± 0.46	0.9998	0.2-25	0.166	0.554
p-Coumaric acid	320	-8.393 ± 4.86	90.350 ± 0.48	0.9998	0.2-25	0.161	0.537

Table 2

Table 2. RSD (%) values of the retention time (RT) and peak area (PA) for each standard.

	Intra-day (n=6)		Inter-day (n=6)	
Compound	RT	PA	RT	PA
Catechin	0.04	1.29	0.02	5.52
Epicatechin	0.03	1.43	0.02	4.14
Quercetin	0.03	0.46	0.04	3.58
Kaempferol	0.02	0.36	0.04	3.38
Q-3-*O*-glucoside	0.17	0.62	0.15	1.19
K-3-*O*-glucoside	0.17	1.46	0.12	1.12
Gallic acid	0.02	0.13	0.26	4.18
Protocatechuic acid	0.14	0.28	0.16	1.38
Caffeic acid	0.04	0.28	0.02	0.78
Ferulic acid	0.01	0.26	0.02	3.42
p-Coumaric acid	0.03	0.20	0.02	1.52

Table 3

Table 3. Mass spectrometry data and concentrations of phenolic compounds in winery by-products of *Vitis vinifera* cv. Zalema

Compound	MS (*m/z*)[1] $[M-H]^-$	MS/MS (*m/z*)[1]	Seeds[2]	Skins[2]	Stems[2]	Pomaces[2]
Flavanols						
Catechin (C)	289	245	65.13±17.06^{a}	17.13±7.82^{b}	57.16±11.58^{a}	37.84±6.30^{c}
Epicatechin (EC)	289	245	43.35±9.14^{a}	4.73±1.02^{b}	10.64±1.97^{c}	13.70±3.28^{c}
Procyanidin B1	577	425, 405, 289	84.61±17.79^{a,c}	32.14±6.52^{b}	88.79±13.90^{a}	72.62±9.73^{c}
Procyanidin B2	577	425, 405, 289	19.09±4.26^{a}	7.50±1.63^{b}	8.31±1.61^{b}	7.59±0.95^{b}
Procyanidin B3	577	425, 405, 289	21.77±8.79^{a}	7.84±2.61^{b}	11.99±2.84^{b}	8.28±3.44^{b}
Procyanidin B4	577	425, 405, 289	31.85±6.37^{a}	15.12±3.35^{b}	33.19±5.69^{a}	23.82±2.29^{c}
Procyanidin B7	577	425, 405, 289	18.25±3.38^{a}	1.77±1.41^{b}	8.22±2.24^{c}	5.51±1.31^{d}
Procyanidin trimer 1	865	577, 289	13.69±2.81^{a}	1.85±0.71^{b}	5.78±1.38^{c}	4.07±1.16^{c}
Procyanidin trimer 2	865	577, 289	72.35±11.29^{a}	7.56±2.31^{b}	13.84±3.45^{c}	23.11±3.98^{d}
Procyanidin tetramer 1	1153	863, 577, 287	26.46±4.94^{a}	9.34±2.19^{b}	27.52±4.78^{a}	18.04±2.04^{c}
Procyanidin tetramer 2	1153	863, 577, 287	0.87±0.66^{a}	1.50±1.26^{a,c}	6.45±1.52^{b}	2.28±0.79^{c}
Galloyled procyanidin	729	577, 425, 407, 289	60.12±9.83^{a}	3.63±1.47^{b}	26.32±4.10^{c}	19.18±3.12^{d}
Procyanidin B2 3-*O*-gallate	729	577, 425, 407, 289	145.62±30.45^{a}	11.79±5.19^{b}	49.97±8.16^{c}	46.61±11.14^{c}
Flavonols						
Quercetin 3-*O*-rutinoside	609	301	0.88±0.34^{a}	10.71±3.07^{b}	3.28±0.49^{c}	8.39±1.27^{d}
Quercetin 3-*O*-glucuronide	477	301	2.51±1.28^{a}	51.01±12.84^{b}	24.74±3.84^{c}	53.70±5.17^{b}
Quercetin 3-*O*-glucoside	463	301	2.72±1.38^{a}	55.26±13.91^{b}	26.81±4.16^{c}	58.18±5.60^{b}
Quercetin pentoside	433	301	0.00±0.00^{a}	0.44±0.21^{b}	0.05±0.15^{a}	0.52±0.09^{b}
Kaempferol hexoside	447	285	0.24±0.24^{a}	4.81±1.56^{b}	1.38±0.22^{c}	3.63±0.65^{d}
Kaempferol 3-*O*-glucoside	447	285	0.81 ± 0.37^{a}	18.75 ± 4.64^{b}	4.97 ± 0.56^{c}	15.15 ± 2.39^{d}
Kaempferol 3-*O*-glucuronide	461	285	n.d.^{a}	n.d.^{b}	0.37 ± 0.28^{c}	0.04 ± 0.00^{d}
Isorhamnetin 3-*O*-glucoside	477	315	0.20±0.07^{a}	2.87±0.86^{b}	1.01±0.15^{c}	2.36±0.35^{d}
Isorhamnetin 3-*O*-glucuronide	491	315	0.63±0.24^{a}	1.80±0.71^{b}	0.82±0.23^{a,c}	1.14±0.37^{c}
Quercetin	301		0.00±0.00^{a}	0.50±0.30^{b}	0.30±0.11^{c}	0.87±0.45^{d}
Kaempferol	285		0.00±0.00^{a}	0.35±0.29^{b}	0.53±0.18^{c}	0.52±0.29^{b,c}
Phenolic acids						
Gallic acid	169	125	16.85±9.01^{a}	3.39±1.44^{b}	12.32±5.81^{a}	12.53±5.97^{a}
Protocatechuic acid	153	109	2.08±0.53^{a}	0.55±0.33^{b}	1.07±0.36^{c}	1.00±0.46^{c}
Caffeic acid	179	135	0.45±0.34^{a}	1.28±0.17^{b}	1.66±0.27^{c}	1.74±0.35^{c}
Caftaric acid	311	179	1.96±0.59^{a}	1.82±0.31^{a}	7.30±2.13^{b}	9.99±3.74^{c}
Fertaric acid	325	193	0.35±0.30^{a}	0.99±0.11^{b}	0.84±0.07^{b,c}	0.80±0.13^{c}
cis-Coutaric acid	295	163	0.64±0.05^{a}	0.73±0.08^{b}	0.68±0.05^{a,b}	0.32±0.03^{c}
trans-Coutaric acid	295	163	0.77±0.09^{a}	0.66±0.08^{a}	1.10±0.18^{b}	1.35±0.69^{b}

[1]Fragment ion detected in negative ion MS/MS.

[2] mg phenolic compound/100 g dry matter. Each value represents mean (n=3) ± SD. Values in the same row followed by different letters are significantly different by ANOVA test ($p<0.05$).

Table 4

Table 4. Total phenolic content and antioxidant activity of by-products of *Vitis vinifera* cv. Zalema

Analysis/By-product	Seeds	Skins	Stems	Pomace
∑Phenols* (mg/100 g)	644.62±90.85[a]	277.77±52.64[b]	437.74±60.77[c]	454.90±37.51[c]
ABTS (mmol TE/100g)	96.64±27.32[a]	42.32±16.03[b]	52.09±6.99[b]	69.47±17.02[c]
FRAP (mmol TE/100g)	38.61±4.61[a]	20.43±6.86[b]	22.14±3.70[b]	35.19±12.55[a]

*∑Phenols: sum of all of individual phenolic compounds.
Each value represents mean (n=3) ± SD. Values in the same row followed by different letters are significantly different by ANOVA test ($p<0.05$).

Figure 1.

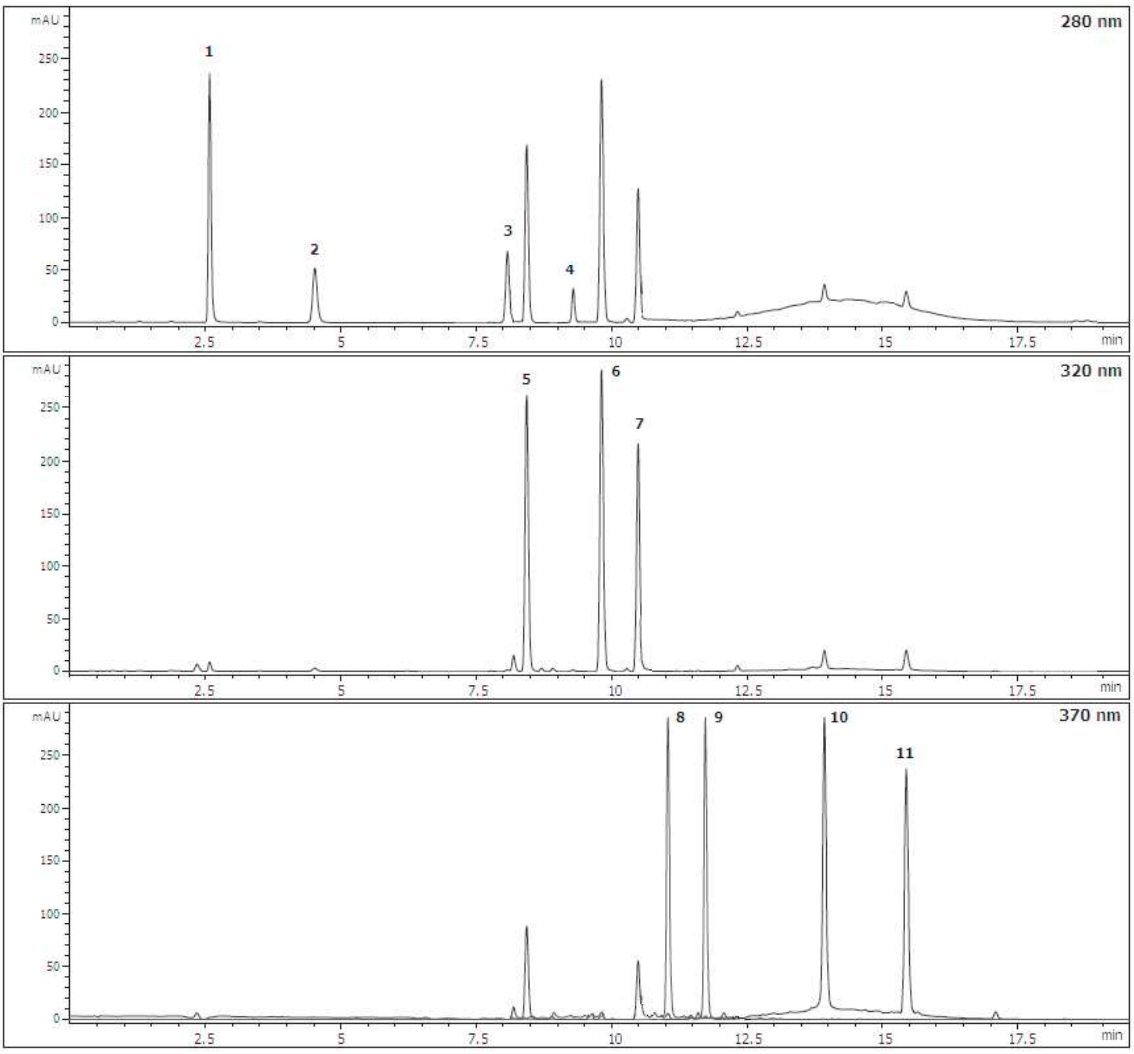
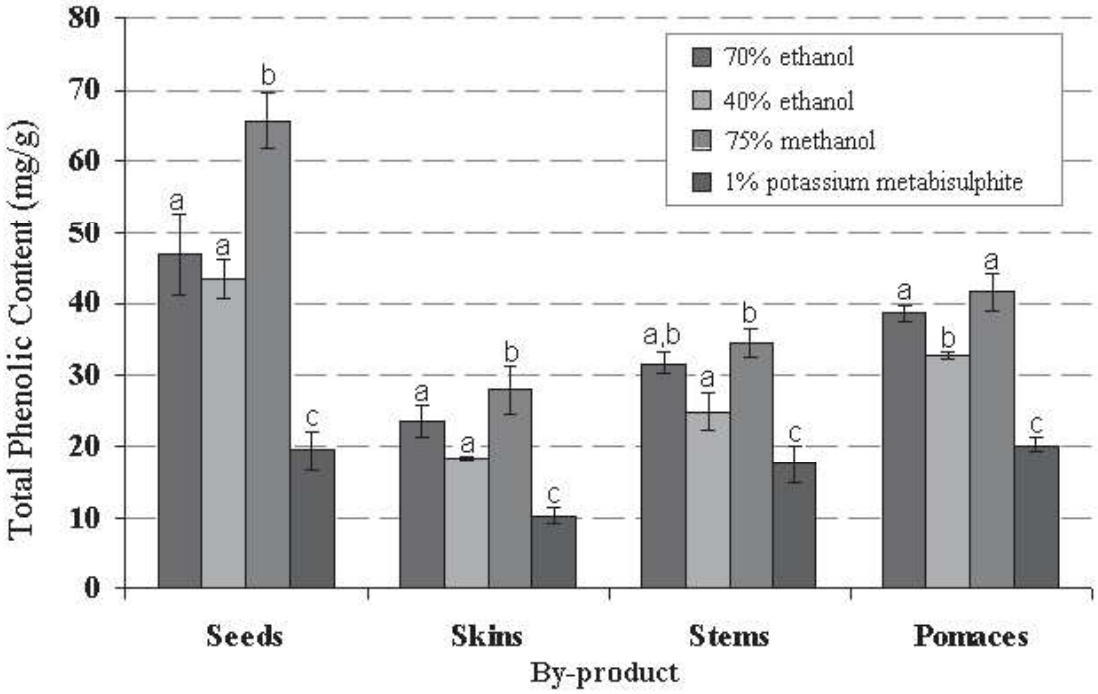


Figure 2.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70
60
50
40
30
20
10
0
Total Phenolic Content (mg/g)
70% ethanol
40% ethanol
75% methanol
1% potassium metabisulphite
a
a
b
c
a
a
b
c
a,b
a
b
c
a
b
a
c
Seeds
Skins
Stems
Pomaces
By-product

Figure 3.

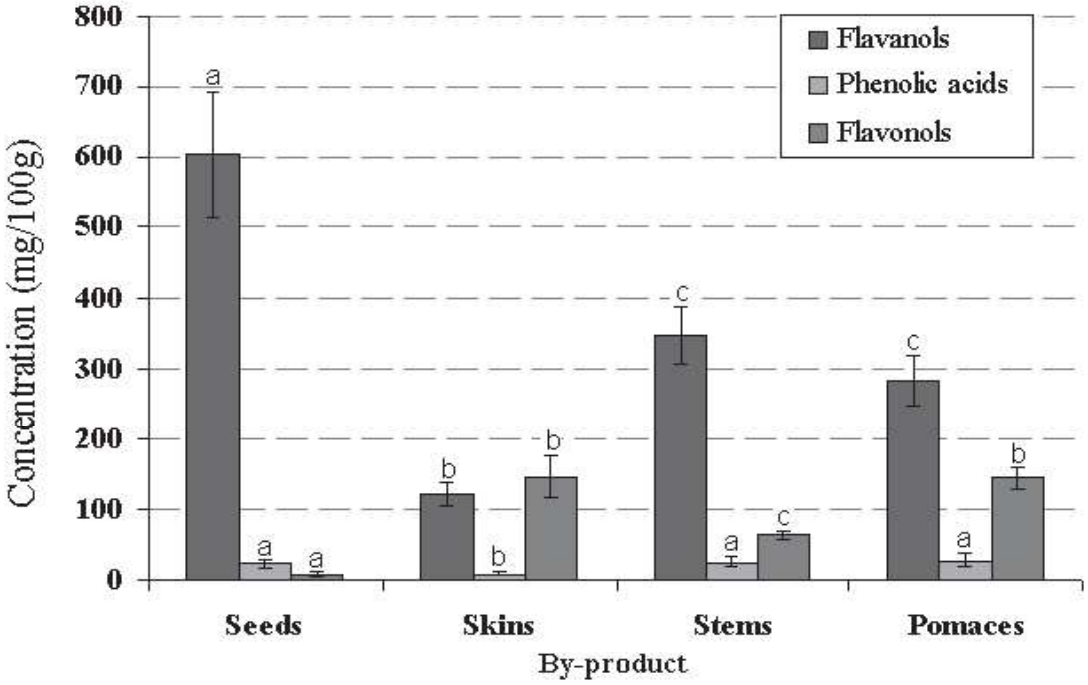
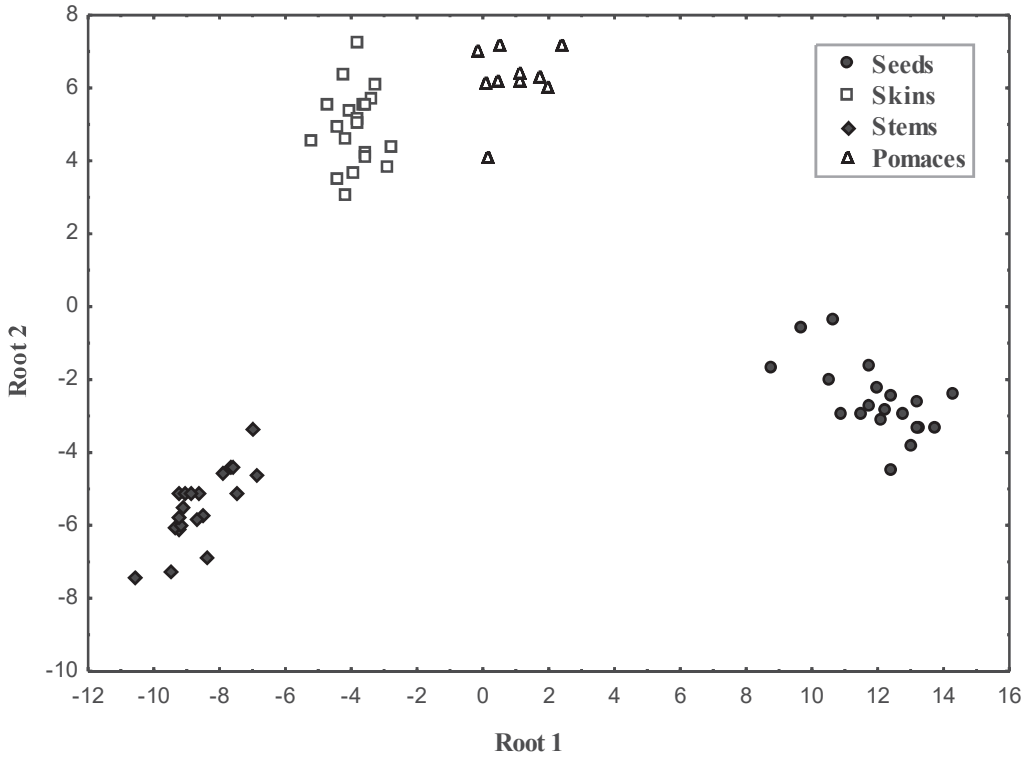

800
700
600
500
400
300
200
100
0
Concentration (mg/100g)
Flavanols
Phenolic acids
Flavonols
a
a
a
b
b
b
c
a
c
c
a
b
Seeds
Skins
Stems
Pomaces
By-product

Figure 4.


Seeds
Skins
Stems
Pomaces
Root 2
Root 1
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-10
-8
-6
-4
-2
0
2
4
6
8
10
12
14
16