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# Injection-port derivatization coupled to GC-MS/MS for the analysis of glycosylated and non-glycosylated polyphenols in fruit samples

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**Running title:** In-port derivatization and GC-MS/MS to analyse polyphenols in fruit.

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**Abstract**

Polyphenols, including glycosylated polyphenols, were analysed via a procedure based on injection-port derivatization coupled to gas chromatography-tandem mass spectrometry (GC-MS/MS). The polyphenols in lyophilized fruit samples were extracted with an acidified MeOH mixture assisted by ultrasound. Samples were dried under vacuum, and carbonyl groups were protected with methoxylamine. Free hydroxyl groups were subsequently silylated in-port. Mass fragmentations of 17 polyphenol and glycosylated polyphenol standards were examined using Multiple Reaction Monitoring (MRM) as the acquisition mode. Furthermore, in-port derivatization was optimized in terms of optimal injection port temperature, derivatization time and sample: *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) volume ratio. A C18 solid-phase-extraction clean-up method was used to reduce matrix effects and injection liner degradation. Using this clean-up method, recoveries for samples spiked at 1 and 10 µg/g ranged from 52 % to 98 %, depending on the chemical compound. Finally, the method was applied to real fruit samples containing the target compounds. The complete chromatographic runtime was 15 min, which is faster than reported for recent HPLC methods able to analyse similar compounds.

## 1. Introduction

Polyphenols, a type of diet-derived anti-oxidant, have received considerable public attention due to their protective effects against cancer and cardiovascular and age-related diseases (Cao, et al., 2008). Classified into anthocyanins, flavones, isoflavones, flavanones, flavonols, and flavanols (Tsao & Yang, 2003), these compounds are found

not only in natural food sources such as fruits (Ignat, Volf, & Popa, 2011), but also in agro-industrial by-products (Delpino-Rius, Eras, Vilaró, Cubero, Balcells, & Canela-Garayoa, 2015) and in beverages, such as tea (Ding, Yang, & Xiao, 1999) and wine (Río Segade, Orriols, Giacosa, & Rolle, 2011).

Polyphenols have been extracted from fruit samples by means of several techniques depending on the sample; most of these include the use of a slightly acidic mixture of aqueous-organic solvents. The extraction is usually assisted by microwave or ultrasound (Picó, 2013). Recently, micro-extraction techniques, which require lower amounts of solvent, have also been used for this purpose (Nerín, Salafranca, Aznar, & Batlle, 2009).

After extraction, the various groups of phenols are commonly analysed by reversed-phase HPLC using a C18 column and UV-vis diode array detector (DAD) (Schieber, Keller, & Carle, 2001). Mass and tandem mass spectrometry play an important role, especially for identification purposes (Campillo, Viñas, Férez-Melgarejo, & Hernández-Córdoba, 2015 and Malec, Le Quéré, Sotin, Kolodziejczyk, Bauduin, & Guyot, 2014). Although HPLC is the primary technique used for the analysis of polyphenols, several studies refer to the analysis of flavonoid aglycones by gas chromatography using silylation to convert the analytes into volatiles (Nolvachai & Marriott, 2013). Examples of this type of analysis can be found using on-column injection (Vinciguerra, Luna, Bistoni, & Zollo, 2003), analysis of polyphenols in apple pomace (Tao, Sun, Chen, Li, Wang, & Sun, 2014) and apple juice (Loots, van der Westhuizen, & Jerling, 2006). Flavonoid aglycones have also been explored in model systems and citrus fruits (Füzfai & Molnár-Perl, 2007) by means of a prior oximation step to obtain a better response, particularly for anthocyanins and apple (Rudell, Mattheis, & Curry, 2008). In addition, a few studies have attempted to analyse flavonoid glycosides as trimethylsilyl (TMS)

derivatives using high temperature chromatography (dos Santos Pereira, Costa Padilha, & Radler de Aquino Neto, 2004). However, these efforts were only qualitative.

Derivatization is often carried out off-line after extraction; however, the possibility has emerged of performing this derivatization on-line, thereby reducing time-consuming sample processing steps, decreasing the amount of reagents, and increasing the analytic speed and efficiency (Docherty & Ziemann, 2001). Among these alternative approaches, on-line processes involving the introduction of the sample and the derivatization reagent directly into the hot GC inlet are known as inlet-based or in-port derivatizations. In this procedure, the derivatization occurs in the gas-phase (Bizkarguenaga, et al., 2013). The sample and the derivatization reagent can be injected separately, either by first manually injecting the sample or the derivatization reagent (Viñas, Martínez-Castillo, Campillo, & Hernández-Córdoba, 2011), requiring the presence of the analyst to start each analysis, or simultaneously, using a software controlled sandwich injection which fills the syringe with both the sample and the derivatization reagent, allowing an air gap between them. The latter is expected to give better results in terms of repeatability and automation of the analytical sequence.

The aim of this work was to develop an injection-port method using a GC-MS/MS instrument, with derivatization performed using an automated sandwich injection of the methoximated sample and the derivatization reagent, namely *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA). In addition, Multiple Reaction Monitoring (MRM) was used for mass acquisition, thus allowing an improvement of the limits of detection. This enhancement is especially useful in the case of glycosylated polyphenols as the derivatization yields of these compounds are generally low, thereby causing lower analyte response. Moreover, the use of electron ionization (EI) as the GC ionization source could provide a mass spectrum with more fragments, which could be a

useful tool for identification purposes. The applicability of this method is demonstrated via the analysis of distinct samples drawn from fruit origins, known to be important sources of polyphenols. To the best of our knowledge, this report describes the first time that a method using GC has been used to analyse polyphenols and glycosylated polyphenols in a single analysis, thus broadening the field of GC applications into analyses traditionally performed by LC. In addition, the chromatographic run time is much faster than current LC methods, requiring only 15 min.

## 2. Material and Methods

### 2.1. Reagents, solvents, and phenolic standards

*N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and methoxylamine hydrochloride (MEOX) were purchased from Sigma-Aldrich (Buchs, Switzerland).

Methanol (MeOH), acetone (HPLC grade purity), ethyl acetate (EtOAc), and pyridine were supplied by J.T. Baker (Deventer, The Netherlands), and water was purified in a Milli-Q system from Millipore (Bedford, MA, USA). Ascorbic acid was purchased from Acros (Pittsburgh, PA, USA) and glacial acetic acid (HAcO) from Panreac (Barcelona, Spain).

Standards of phenolic compounds were supplied as follows: (+)-catechin, (−)-epicatechin, procyanidin B1, procyanidin B2, quercetin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-rutinoside,

kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside, epigallocatechin gallate, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside by Extrasynthèse (Genay, France), and phloretin-2′-*O*-β-glucoside and 5′-caffeoylquinic acid by Sigma-Aldrich Chemie (Steinheim, Germany). Standard stock solutions of 100 µg/mL of phenolic compounds were prepared in MeOH and stored at −80 °C in amber glass vials. Working solutions of 50 and 10 µg/mL were prepared from stock solutions by sampling an aliquot and diluting as necessary with MeOH.

A C18 SepPak® cartridge (400 mg packing, Waters, Milford, MA, USA) sorbent was used for solid-phase extractions (SPEs). A Visiprep SPE vacuum manifold from Supelco (Bellefonte, PA, USA) was used to process up to 12 SPE tubes simultaneously.

## 2.2. Instrumentation

The GC-MS/MS analyses were performed with an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) with a multimode injector and a splitless liner containing a piece of glass wool. A fused silica high-temperature capillary column (J&W DB–1HT, 15 m × 0.32 mm i.d.; 0.10 µm film thickness) from Agilent was used at constant pressure. The detector was an Agilent 7000B triple quadrupole mass spectrometer with an inert EI ion source. The mass spectrometer worked in MRM mode with the EI ionization source at 70 eV. Helium with a purity of 99.9999 % was used as both the carrier and quenching gas, and nitrogen with a purity of 99.999 % as the collision gas, both supplied by Air Liquide (Madrid, Spain).

For control and data analysis, Agilent Mass Hunter B.04.00 software was used.

119

120 2.3. Samples

121

122 Randomly chosen Golden Delicious and Royal Gala apples, Blanquilla pears, and red
123 plums were purchased from a local market (approximately 1 kg of each). In addition,
124 processed foodstuffs of fruit origin, namely apple juice concentrate, natural peach juice,
125 apple/peach juice, raspberry jam, and cranberry juice were supplied by local industries.
126 Fruits were homogenized in a blender (Grindomix GM 200; Retsch, Haan, Germany) at
127 5000 rpm for 2 min, and ascorbic acid (~ 10 g/kg) was added to prevent oxidation.
128 Samples were immediately frozen at −80 °C and lyophilized at −50 °C and 1.1 Pa for 24
129 h in a Cryodos-50 lyophilizer (Telstar, Terrassa, Spain). Processed foodstuff samples
130 were frozen at −80 ºC before being lyophilised. Finally, the lyophilised samples were
131 powdered and stored at −20 ºC until analysis.

132

133 2.4. Analytical procedure

134

135 Approximately 100 mg of each of the lyophilised samples was placed into 15-mL
136 polypropylene tubes. Subsequently, 2 mL of a $H_2O$-methanol (20:80) solution acidified
137 with 1 % of HAcO was added to each tube. The mixture was subjected to an ultrasonic
138 bath (ATU APM40-2LCD; Madrid, Spain) for 10 min followed by 20 min of vortex
139 agitation and centrifugation at 1400×*g* for 10 min (Hettich Eppendorf Centrifuge
140 MIKRO 22 R; Tuttlingen, Germany). A 1 mL volume of the extract was made up with 3
141 mL of deionized $H_2O$ before SPE clean-up.

142 The C18 classic SepPak cartridge was first conditioned with 3 mL of methanol followed
143 by 2 mL of $H_2O$ (1 % HAcO v/v). The sample extract was then applied to the cartridge.
144 Co-extracted substances (e.g., sugars and organic acids) were rinsed from the sorbent
145 with $H_2O$ (acidified at 1 %, v/v with HAcO). Subsequently, the cartridge was eluted
146 with 1.5 mL of methanol (1 % HAcO v/v) followed by 0.5 mL of EtAcO. The solvents
147 were evaporated under reduced pressure at room temperature using a SpeedVac
148 (Thermo, Asheville, NC, USA). The residue was dissolved in 300 µl of a solution of
149 MEOX in pyridine (20 mg/mL) and incubated at 45 ºC for 1 h in a ThermoMixer
150 (Eppendorf AG, Hamburg, Germany). Prior to injection into the gas chromatograph, the
151 methoximated sample was placed in a chromatography vial containing a glass insert.

152 Sandwich injection of the sample and the derivatization reagent (MSTFA) in a ratio of
153 2:3 µl was carried out in splitless mode applying an inlet temperature program as
154 follows: 100 ºC (held for 3 min), then increased to 320 ºC at 250 ºC/min. The GC oven
155 temperature was programmed as follows: 70 ºC (held for 3 min), then increased to 270
156 ºC at 50 ºC/min, and then to 340 ºC at 10 ºC/min (held for 1 min) at a constant pressure
157 of 10.31 psi. A 5-min backflush using a restrictor (0.7 m x 150 µm) inert capillary
158 column at 340 ºC and 60 psi was programmed after each run to eliminate the
159 compounds retained in the chromatographic column. These compounds result from the
160 incomplete derivatization of some of the low volatility analytes. Moreover, the vial cap
161 of the derivatization reagent was replaced every 10 injections to prevent contamination
162 from the vial septum.

163 The temperatures of the ion source and the transfer line were 250 ºC and 300 ºC,
164 respectively. An MRM method was created keeping the temperature of both
165 quadrupoles at 150 ºC. Two transitions were monitored for each analyte, the first for
166 quantification and the second for confirmation. Table 1 shows the selected mass

spectrometer conditions. The resolution was adjusted to 1.0 Da for quadrupoles 1 and 3. The solvent delay was 5 min.

## 3. Results and Discussion

### 3.1. Optimization of the chromatographic and MS/MS conditions

The chromatographic conditions were optimized using a standard mixture to achieve the efficient separation of the 17 target compounds (see conditions in section 2.2) in a 15 min run—shorter than current HPLC methods (Díaz-García, Obón, Castellar, Collado, & Alacid, 2013, Fischer, Carle, & Kammerer, 2011, Castellar, Collado, & Alacid, 2013 and Fischer, Carle, & Kammerer, 2011). Quantitation parameters for all compounds are listed in Table 1.

Using the non-polar J&W DB-1HT column, the retention times of the target compounds increased with the number of TMS groups. This behaviour has been previously described in polyphenol studies, which have been analysed with similar non-polar columns (Gao, Williams, Woodman, & Marriott, 2010 and Koupai-Abyazani, Creaser, & Stephenson, 1992). Hence, higher retention times were observed for the compounds with a disaccharide as the glycosidic unit versus those that had a monosaccharide unit for polyphenols with the same aglycone. Moreover, aglycones had lower retention times compared with the former two. Those studies also reported the retention order of TMS silylated polyphenols with the same substituents as flavan-3-

ol<chalcone<flavonone<isoflavone<flavonol<flavone. On this basis, the retention time of anthocyanins appears to be close to that of chalcones.

### 3.2. Optimization of in-port derivatization

Trimethylsilyl derivatives are routinely used in GC to increase the volatility and thermal stability of organic compounds carrying hydroxyl groups. In this study, a prior methoximation of the dry sample was performed in order to protect the carbonyl groups—present in many of the structures—and to enhance the derivatization yield of the compounds. Moreover, the aprotic nature of pyridine, as a solvent that solubilizes derivatives, protects the target analytes against hydrolysis. EtAcO and hexane were also tested as alternatives to pyridine. The response obtained with these solvents diminished (data not shown).

Following methoximation, the silylation conditions of the methoximated extract prepared from a standard mixture of all target compounds were optimized in terms of time (purge off), temperature (Figure 1a), and sample volume/MSTFA ratio (Figure 1b). In this study, the sample and the derivatization reagent were sandwich injected simultaneously. Previous studies reported optimum temperatures approximately 200 ºC for the in-port derivatization of compounds, such as (+)-catechin and (-)-epicatechin (Viñas et al., 2011). However, at this temperature, a peak at the retention time of the quercetin aglycone was observed for glycosylated polyphenols, such as quercetin glycosides. This finding could be attributable to the breakage of the glycosylic bond, thus yielding a signal for quercetin. Therefore, temperatures between 70 ºC and 150 ºC were tested for the in-port derivatization of glycosylated polyphenols. This temperature

range did not affect the method performance, as shown by a high response for aglycones. The best results were obtained at 100 ºC. The derivatization time ranged between 0.5 and 5 min, yielding maximum performance at 3 min (Figure 1a). Using these optimum conditions, the ratio of methoximated extract versus MSTFA volume was optimized using 1:1, 1:2, 2:1, 2:3 and 3:2 volume ratios. Similar results were obtained for 2:1 and 2:3 ratios using standards (Figure 1b). Analysis of variance (ANOVA) showed that the most significant parameters in the optimization of in-port derivatization at a 95 % confidence level were injection temperature and sample:derivatization reagent ratio. As it is expected that the matrix may play a role in the optimum ratio, both conditions were further studied in real sample matrices of the fruits under study.

Sample matrices of different origin were spiked with all the target compounds at a concentration between 1 and 10 µg/mL and injected into the GC system using the two selected ratios for standards, namely sample:MSTFA volume ratios of 2:1 and 2:3. Figure 2 shows that the second condition led to a marked improvement in the detection of polyphenols. This enhancement was especially high for most of the compounds in raspberry jam. This could be attributable to this matrix containing a higher amount of sugars, which interfere with the derivatization reaction of the target analytes. On the other hand, a considerable decrease in response for phloretin 2′-*O*-glucoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-rutinoside was observed in apple samples compared with the other two fruit samples, likely due to the higher content of organic acids in this matrix. Although a decrease was noticed for these three compounds in a certain matrix, in general a considerable increase was observed in the responses for most of the compounds when using a 2:3 ratio. This finding may be attributable to the fact that fruit matrices contain a high concentration of co-extractives, such as sugars and organic

238 acids, which reduce the derivatization efficiency of polyphenols. Consequently, a
239 sample:MSTFA volume ratio of 2:3 was used for further analyses.

240

241 3.3. Method performance

242

243 The performance parameters of the GC-MS/MS method for the optimized conditions
244 described in sections 3.1 and 3.2 were evaluated in terms of LOD, LOQ, and intra- and
245 inter-day repeatability (expressed as relative standard deviation), correlation coefficient
246 (r) and linear range as summarized in Table 2. In this regard, LOD and LOQ were
247 calculated as the concentrations giving S/N=3 and S/N=10, respectively, for standard
248 solutions (due to the impossibility of spiking blank fruit samples, as they are natural
249 sources of the target compounds). Because of this, instrumental limits were lower for
250 low molecular weight compounds, namely aglycones, with LODs between 6-30 ng/mL
251 and LOQs between 20-100 ng/mL, increasing for those with a monosaccharide as a
252 glycoside unit and for those with a disaccharide as a glycoside unit (LOD<240,
253 LOQ<800 ng/mL), which in the case of the studied target compounds was rutinose. The
254 higher LOD and LOQ values obtained for higher molecular weight compounds could be
255 attributed to a lower derivatization yield due to the high number of hydroxyl groups
256 present in these molecules, with their consequent steric hindrance. We have previously
257 assayed off-line derivatization for glycosylated polyphenols, observing similar
258 behaviour (data not shown). The LODs for aglycones were very similar to those
259 reported in modern HPLC-DAD methodologies (≤20 ng/mL) (Abad-García, Berrueta,
260 López-Márquez, Crespo-Ferrer, Gallo, & Vicente, 2007) and better than observed with
261 other previously published methods (Tsao & Yang, 2003). Although the efficiency of

262 the derivatization decreases with the molecular weight, the described methodology
263 showed higher LODs than HPLC for glycosylated polyphenols, which for HPLC are
264 approximately 30 ng/mL using modern methods, and very similar or even better than
265 those reported by Tsao and Yang (2003). Repeatability was studied at two concentration
266 levels of the methoximated extract (1 µg/mL and 5 µg/mL). Upright %RSD values were
267 obtained for intra- and inter-day repeatability, ranging from 3 % to 12 % and from 5 %
268 to 18 %, respectively. Repeatability values are better for aglycones (<9 %), and they
269 generally increase with the molecular weight due to a loss of the derivatization
270 efficiency. Correlation coefficient values ranged from 0.973 to 0.999. The linear range
271 in which calibration curves were studied showed the same behaviour as reported for the
272 limits of detection, where lower molecular weight species, namely (-)-epicatechin, (+)-
273 catechin and 5-caffeoylquinic acid allowed a greater linear range than glycosylated
274 species.

275

276 3.4. Matrix effects

277

278 In gas chromatography, matrix effects may occur in the injection port, where the
279 derivatization reaction takes place. Furthermore, the analytes of the matrix include
280 many co-extractives, mainly carbohydrates and organic acids, which compete for the
281 derivatization reagent. Matrix effects were therefore studied in order to determine the
282 feasibility of using an external standard calibration curve to quantify the analytes. The
283 matrix effects were assessed in three matrices of distinct origin (apple fruit, red plum
284 fruit, and raspberry jam) and were calculated by comparing the signal response obtained
285 when spiking a sample after extraction (at 1 µg/mL for aglycones and at 10 µg/mL for

286 glycosylated polyphenols) with the signal response obtained from a standard solution at
287 the same concentration (Eq. 1).

288

289 $$\%\ \mathrm{ME} = \left(\frac{\text{Area post extraction spiked sample}}{\text{Area of standard}} - 1\right) \times 100 \quad (1)$$

290

291 A non-spiked sample was also analysed for each of the matrices in order to subtract the
292 signal produced for compounds already present in the sample. For all the matrices, the
293 signal considerably decreased for compounds that gave a lower response, namely
294 glycosylated flavonols, anthocyanins, and procyanidin dimers, with the decrease being
295 especially noticeable for the first two. This can be explained by the fact that although an
296 increase in the ratio of derivatization reagent in the injection port gave an increase in the
297 response, it was not enough to achieve a response equivalent to the same concentration
298 of the compounds in the standard solution. In contrast, (-)-epicatechin, (+)-catechin, and
299 5-caffeoylquinic acid showed the opposite behaviour, giving a slight signal
300 enhancement (<28 %). In general, enhancement could be attributed to the presence of
301 co-extractives, which mask the active sites in the chromatographic system, resulting in
302 lower adsorption of the analytes (generally in the liner) resulting in signal enhancement.

303 Moreover, the reproducibility of the derivatization reaction in the different matrices
304 over time under these conditions was not consistent, most likely because the injection of
305 a large amount of matrix components caused gradual accumulation of non-volatile
306 components in the GC system, resulting in the formation of new active sites and a
307 gradual decrease in analyte response (Rahman, Abd El-Aty, & Shim, 2013). According
308 to Schenck et al., two opposing phenomena should be considered when studying matrix
309 effects in GC. One is the degree of enhancement of the analyte response after repeated

310 injections. The second is decreases in the responses as a result of a dirty injection liner
311 (Schenck & Lehotay, 2000). Considering these concerns, a clean-up step to reduce these
312 effects was studied.

313

314 3.4.1. SPE clean-up

315

316 In order to reduce the matrix effects and simultaneously improve the reproducibility of
317 sample analysis, a clean-up step using a C18 SepPak was introduced into the analytical
318 method (Wrolstad, et al., 2005). After being conditioned, the column was loaded with
319 the sample and washed with aqueous acid solution to remove carbohydrates and organic
320 acids. Finally, polyphenols were eluted with 1.5 mL of methanol (acidified at 1 %, v/v
321 with HAcO). Note that in some studies a 0.1 % HCl solution is used to elute
322 polyphenols. The acid tends to stabilize polyphenols, especially anthocyanins; however,
323 it can also cause acid hydrolysis during concentration to dryness (Wrolstad et al., 2005),
324 which is an essential step in GC analyses of the nature reported here. Consequently, a
325 weaker acid, HAcO, was used. To assess the suitability of performing a clean-up step
326 with a C18 cartridge, three matrices under study (apple fruit, red plum fruit, and
327 raspberry jam) were spiked before performing the SPE and the areas obtained for the
328 target polyphenols compared with those obtained for standard mixtures at the same
329 concentration. Table 3 shows that after applying the SPE clean-up, the signal improved
330 for all analytes (except cyanidin-3-*O*-rutinoside, which showed a slight decrease)
331 compared to the response obtained without this clean-up. Matrix effects for the other
332 compounds showed an enhancement generally below 20 %, except for phloretin 2′-*O*-
333 glucoside, which increased to 60 %. Given that matrix effects were highly reduced and

334 controlled with the use of SPE, calibration by external calibration curve was used
335 because good correlation values were obtained for most of the analytes (Table 2). In
336 addition, the alternatives to this calibration would have been standard addition, which is
337 time-consuming and labour intensive, or the use of an internal standard, which was not
338 suitable because the derivatization and analytical performance of each of the target
339 compounds is very different.

340 Recoveries were assessed in three spiked matrices (at 1 µg/mg for aglycones and at 10
341 µg/mg for glycosylated polyphenols) applying the C18 SPE clean-up. Moreover, two
342 elution solvent combinations were studied to enhance recoveries after the clean-up
343 (Table 3). The main drawback of this clean-up step was that 5-caffeoylquinic acid
344 showed low recoveries (approximately 50 %) as it was partially washed off the column
345 with $H_2O$. Recoveries for the other target compounds ranged from 66 % to 92 % and
346 from 77 % to 96 % when using methanol and methanol-EtOAc, respectively.
347 Recoveries generally improved when the elution with acidified methanol was followed
348 by EtOAc. This improvement was especially noticeable for the most apolar compounds,
349 such as flavonols and flavonol glycosides, whereas anthocyanins and flavan-3-ols
350 showed little improvement. Consequently, the combination of 1.5 mL of MeOH and 0.5
351 mL of EtOAc was selected for further analyses.

352

353 3.6. Application to samples

354

355 The optimized methodology was applied to determine 17 target polyphenols in Golden
356 Delicious and Royal Gala apples, Blanquilla pears, and plum fruit as well as in
357 processed foods of fruit origin, namely, apple juice concentrate, natural peach juice

from the Clingstone cultivar, a mixture of apple and peach juice, raspberry jam, and cranberry juice (Table 4). All samples were analysed in triplicate.

All of the samples contained the flavan-3-ols (-)-epicatechin and (+)-catechin, which were present at concentrations ranging from 1.3 to 2413.1 µg/g. A strong relationship was observed between these compounds and their corresponding dimers, namely procyanidin B2 and procyanidin B1, as previously reported in peach (Scordino, Sabatino, Muratore, Belligno, & Gagliano, 2012) and apple (Tsao, Yang, Young, & Zhu, 2003) samples. In addition, procyanidin B2 was the compound found at the highest concentration (5187.3 µg/g) in a plum fruit sample. Although 5-caffeoylquinic acid gave lower recoveries, most of the samples showed high concentrations of this compound (1.9-4350.4 µg/g). Variable concentrations of quercetin glycosides were detected only in apple and plum fruit, raspberry jam, and cranberry juice. In samples containing flavonol glycosides, aglycone quercetin was consistently present, although generally at very low concentrations (<5.4 µg/g). The other flavonols, namely isorhamnetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside, were detected only in peach juice products, at low concentrations. The dihydrochalcone phloretin 2′-*O*-glucoside was found only in apple-based products, both fresh fruit and juice. This result is consistent with that reported by several authors (Spanos, Wrolstad, & Heatherbell, 1990). Anthocyanins were found only in samples which had been previously reported in the literature, such as raspberry jam, cranberry juice, plum fruit, peach juice, and Royal Gala apple (Welch, Wu, & Simon, 2008). The content fluctuated noticeably, ranging from traces up to 58.5 µg/g and from 0.1 to 337.4 µg/g for cyanidin-3-*O*-galactoside and cyanidin-3-*O*-glucoside, respectively. Cyanidin-3-*O*-rutinoside was detected only in plum fruit but at a concentration of 57.9 µg/g. These results support the theory that food processing causes the degradation of polyphenols (Kahle, Kraus, & Richling, 2005). In

this regard, the content of flavonols and flavan-3-ols decreased considerably between fresh apple fruit and an apple juice concentrate. In the peach juice, which was prepared by squeezing rather than from concentrate, such as the apple juice, the polyphenol content was higher, giving notable concentrations of flavan-3-ols, flavonols, and anthocyanins.

## 4. Conclusions

An analytical method consisting of injection-port derivatization coupled to gas chromatography-tandem mass spectrometry was developed to determine 17 target polyphenols, including glycosylated polyphenols, in various fruit matrices. The chromatographic separation of the compounds was achieved in only 15 min, which is faster than reported for recent HPLC methods able to analyse similar compounds. Injection-port derivatization was optimised at 3 min and 100 ºC with a 2:3 sample:derivatization reagent ratio. LOD and LOQ were assessed for the target compounds, giving values below 240 and 800 ng/mL, respectively. Repeatability (%RSD at 1 µg/mL and 10 µg/mL, n=5) was below 18 % for all the target compounds. In addition, a clean-up step with a C18 SPE cartridge was necessary to reduce matrix effects produced by the high abundance of sugars and organic acids co-extracted with the target compounds and to prevent the rapid deterioration of the injection liner. Finally, the method was applied to various fruit samples that are known sources of the target compounds. The polyphenol contents of the samples ranged from traces up to 5187.3 µg/g (procyanidin B2 in plum fruit). To summarize, this method offers a new

406 and fast alternative to HPLC to analyse target polyphenols in several fruit samples,
407 which is of great interest in food science.

408

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411

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## Tables

**Table 1:** GC-MS/MS retention time and selected transitions for the target polyphenols.

| compound | r.t. (min) | precursor ions (*m/z*) | product ions (*m/z*)[a] | collision energy (eV)[a] |
|---|---|---|---|---|
| (-)-epicatechin | 7.82 | 368 | 249, 265 | 20 |
| (+)-catechin | 7.89 | 368 | 249, 265 | 20 |
| 5-caffeoylquinic acid | 8.23[b]/ 8.59 | 345 | 73, 255 | 35, 20 |
| quercetin | 8.72 | 647 | 73, 575 | 60, 50 |
| cyanidin-3-*O*-galactoside | 9.67 | 382 | 73, 355 | 20, 35 |
| cyanidin-3-*O*-glucoside | 9.79 | 382 | 73, 355 | 20, 35 |
| phloretin 2′-*O*-glucoside | 9.83 | 342, 547 | 327, 179 | 20, 20 |
| quercetin-3-*O*-galactoside | 10.78 | 647 | 73, 576 | 60, 50 |
| quercetin-3-*O*-glucoside | 10.89 | 647 | 73, 559 | 60, 60 |
| quercetin-3-*O*-rhamnoside | 11.12 | 647 | 73, 560 | 60, 50 |
| epigallocatechin gallate | 11.38 | 369 | 179, 281 | 35, 20 |
| cyanidin-3-*O*-rutinoside | 12.38 | 382 | 73, 355 | 20, 35 |
| procyanidin-B2 | 12.54 | 368 | 249, 191 | 20, 20 |
| procyanidin-B1 | 12.60 | 368 | 249, 191 | 20, 20 |
| kaempferol-3-*O*-rutinoside | 12.76 | 502 | 487, 415 | 20, 50 |
| quercetin-3-*O*-rutinoside | 13.04 | 590, 575 | 575, 503 | 20, 50 |
| isorhamnetin-3-*O*-rutinoside | 13.06 | 532 | 517, 487 | 20, 50 |

[a] Underlined values were used for quantification transitions.

[b] For 5-caffeoylquinic acid peaks corresponding to the two oximes formed during methoximation were observed.

534 **Table 2:** Quality parameters for the analysis of polyphenol standards.

| | | | intra-day repeatability (% RSD, n=5) | | inter-day repeatability (% RSD, n=5) | | correlation coefficient | linear range |
|---|---|---|---|---|---|---|---|---|
| **compound** | **LOD (ng/mL)** | **LOQ (ng/mL)** | **1 µg/mL** | **5 µg/mL** | **1 µg/mL** | **5 µg/mL** | **(r)** | **(µg/mL)** |
| (-)-epicatechin | 6 | 20 | 7 | 5 | 5 | 7 | 0.995 | 0.020-5.1 |
| (+)-catechin | 6 | 20 | 6 | 4 | 7 | 6 | 0.995 | 0.020-5.9 |
| 5-caffeoylquinic acid | 15 | 50 | 8 | 3 | 9 | 8 | 0.994 | 0.050-6.8 |
| quercetin | 30 | 100 | 7 | 4 | 9 | 7 | 0.999 | 0.163-10.4 |
| cyanidin-3-*O*-galactoside | 30 | 100 | 8 | 6 | 12 | 10 | 0.982 | 0.114-14.4 |
| cyanidin-3-*O*-glucoside | 30 | 100 | 9 | 5 | 14 | 11 | 0.986 | 0.114-17.2 |
| phloretin 2′-*O*-glucoside | 30 | 100 | 7 | 4 | 10 | 8 | 0.990 | 0.114-11.2 |
| quercetin-3-*O*-galactoside | 240 | 800 | 10 | 6 | 12 | 10 | 0.992 | 0.866-13.8 |
| quercetin-3-*O*-glucoside | 240 | 800 | 11 | 7 | 15 | 10 | 0.980 | 0.772-12.4 |
| quercetin-3-*O*-rhamnoside | 240 | 800 | 11 | 6 | 15 | 10 | 0.998 | 0.833-13.4 |
| epigallocatechin gallate | 100 | 300 | 8 | 4 | 11 | 9 | 0.973 | 0.174-11.2 |
| cyanidin-3-*O*-rutinoside | 30 | 100 | 8 | 4 | 12 | 9 | 0.988 | 0.114-7.2 |
| procyanidin-B2 | 15 | 50 | 7 | 5 | 9 | 9 | 0.990 | 0.026-6.8 |
| procyanidin-B1 | 15 | 50 | 7 | 3 | 8 | 7 | 0.992 | 0.043-5.6 |
| kaempferol-3-*O*-rutinoside | 180 | 600 | 10 | 6 | 12 | 10 | 0.997 | 0.452-13.8 |
| quercetin-3-*O*-rutinoside | 240 | 800 | 12 | 7 | 18 | 14 | 0.982 | 0.864-13.8 |
| isoharmnetin-3-*O*-rutinoside | 100 | 300 | 9 | 6 | 11 | 8 | 0.997 | 0.362-8.4 |

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538 **Table 3:** M.E (%) and recoveries (%) obtained in three matrices spiked at 1 µg/mg for aglycones and at
539 10 µg/mg for glycosylated polyphenols.

| | M.E. (%) | | Recoveries (%) | |
|---|---|---|---|---|
| **compound** | **without SPE clean-up** | **with SPE clean-up** | **2 mL MeOH** | **1,5 mL MeOH + 0,5 mL EtOAc** |
| (-)-epicatechin | 10 | 13 | 92 | 96 |
| (+)-catechin | 11 | 14 | 90 | 94 |
| 5-caffeoylquinic acid | 20 | 30 | 49 | 52 |
| quercetin | -36 | 30 | 66 | 84 |
| cyanidin-3-*O*-galactoside | -70 | 19 | 78 | 83 |
| cyanidin-3-*O*-glucoside | -70 | 23 | 76 | 77 |
| phloretin 2′-*O*-glucoside | -65 | 63 | 80 | 98 |
| quercetin-3-*O*-galactoside | -83 | 32 | 68 | 80 |
| quercetin-3-*O*-glucoside | -72 | 23 | 72 | 83 |
| quercetin-3-*O*-rhamnoside | -67 | 23 | 75 | 88 |
| epigallocatechin gallate | -51 | -9 | 71 | 91 |
| cyanidin-3-*O*-rutinoside | -86 | 10 | 80 | 85 |
| procyanidin-B2 | -19 | 8 | 88 | 88 |
| procyanidin-B1 | -20 | 6 | 76 | 82 |
| kaempferol-3-*O*-rutinoside | -37 | 20 | 75 | 86 |
| quercetin-3-*O*-rutinoside | -47 | 13 | 78 | 89 |
| isorharnetin-3-*O*-rutinoside | -52 | 12 | 80 | 92 |

540 % RSD (n=3) <25%.

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**Table 4:** Sample analysis expressed as µg/g (dry weight).

| compound | golden delicious | royal gala | pear | plum fruit | apple juice | peach juice | apple/ peach juice | raspberry jam | cranberry juice |
|---|---|---|---|---|---|---|---|---|---|
| (-)-epicatechin | 748.7 ± 3.0 | 962.7 ± 19.3 | 2.4 ± 0.5 | 2413.1 ± 48.3 | 4.6 ± 0.8 | 14.6 ± 1.9 | 7.7 ± 1.5 | 153.5 ± 5.2 | 58.5 ± 8.5 |
| (+)-catechin | 27.2 ± 4.1 | 52.8 ± 4.2 | <LOQ | 277.3 ± 13.9 | 1.3 ± 0.3 | 177.0 ± 22.8 | 86.4 ± 8.6 | 4.0 ± 1.2 | 3.0 ± 0.6 |
| 5-caffeoylquinic acid | 3763.1 ± 75.3 | 4350.4 ± 130.5 | 20.5 ± 0.6 | 127.6 ± 16.6 | 868.7 ± 29.5 | 3589.8 ± 71.8 | 2514.7 ± 186.1 | 1.9 ± 0.5 | 131.3 ± 21.5 |
| quercetin | <LOQ | 5.4 ± 1.6 | <LOQ | 6.9 ± 0.7 | <LOQ | <LOQ | <LOQ | <LOQ | 39.1 ± 6.3 |
| cyanidin-3-*O*-galactoside | n.d. | 53.5 ± 2.7 | n.d. | 89.6 ± 4.7 | n.d. | n.d. | <LOQ | 13.0 ± 2.1 | 29.2 ± 10.8 |
| cyanidin-3-*O*-glucoside | n.d. | n.d. | n.d. | 337.4 ± 32.1 | n.d. | 1.5 ± 0.2 | 1.1 ± 0.3 | 33.2 ± 4.2 | n.d. |
| phloretin 2′-*O*-glucoside | 455.2 ± 45.5 | 113.8 ± 13.7 | n.d. | n.d. | 34.7 ± 7.3 | n.d. | 7.3 ± 2.0 | n.d. | n.d. |
| quercetin-3-*O*-galactoside | 2.8 ± 0.7 | 58.2 ± 3.7 | n.d. | 3.4 ± 0.7 | n.d. | n.d. | <LOQ | n.d. | 58.5 ± 7.4 |
| quercetin-3-*O*-glucoside | n.d. | <LOQ | n.d. | <LOQ | n.d. | n.d. | <LOQ | <LOQ | <LOQ |
| quercetin-3-*O*-rhamnoside | 9.1 ± 0.39 | 20.6 ± 2.5 | n.d. | 47.5 ± 14.0 | <LOQ | n.d. | <LOQ | 3.3 ± 0.4 | 26.2 ± 4.4 |
| epigallocatechin gallate | n.d. | n.d. | n.d. | n.d. | n.d. | <LOQ | <LOQ | <LOQ | n.d. |
| cyanidin-3-*O*-rutinoside | n.d. | n.d. | n.d. | 152.3 ± 7.8 | n.d. | n.d. | n.d. | n.d. | n.d. |
| procyanidin-B2 | 985.6 ± 39.4 | 1024.4 ± 30.7 | n.d. | 5187.3 ± 544.7 | <LOQ | 490.1 ± 16.7 | 10.0 ± 1.4 | n.d. | 34.6 ± 7.6 |
| procyanidin-B1 | 268.3 ± 34.9 | 270.0 ± 43.2 | n.d. | 231.0 ± 37.2 | n.d. | 23.3 ± 2.8 | 317.2 ± 67.6 | n.d. | n.d. |
| kaempferol-3-*O*-rutinoside | n.d. | n.d. | n.d. | <LOQ | n.d. | 4.3 ± 0.5 | 2.7 ± 67.6 | n.d. | n.d. |
| quercetin-3-*O*-rutinoside | n.d. | n.d. | n.d. | 57.9 ± 3.1 | n.d. | n.d. | n.d. | n.d. | n.d. |
| isorhamnetin-3-*O*-rutinoside | n.d. | n.d. | n.d. | n.d. | n.d. | 10.1 ± 1.0 | 4.7 ± 1.4 | n.d. | n.d. |

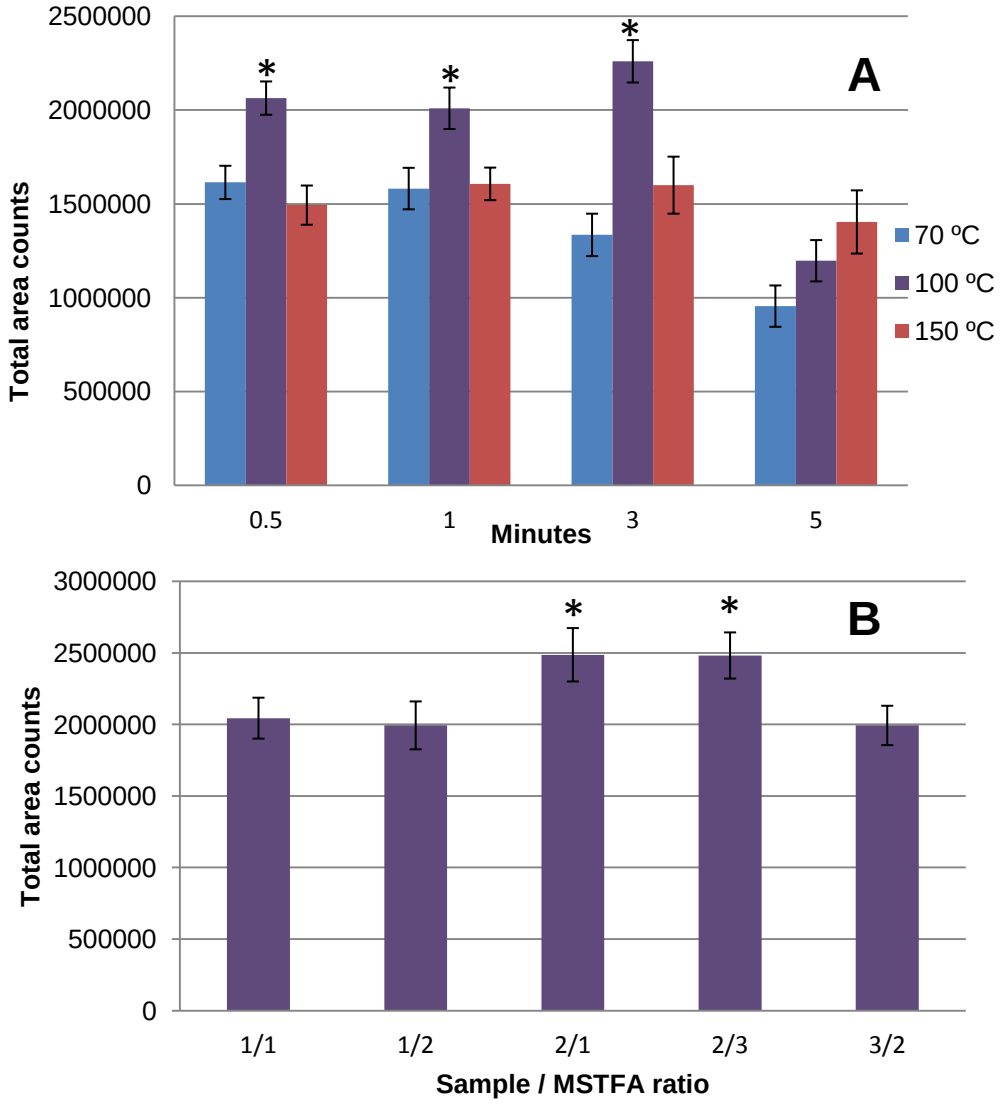
Values are mean ± standard deviation (n=3).

n.d.: not detected; <LOQ: detected but with a S/N<10.

**Figure captions**

**Figure 1**: Optimization of in-port derivatization in terms of: A) temperature and time and B) sample:MSTFA volume ratio. *: Shows conditions found to be statistically different (95% confidence level):

**Figure 2:** Effect of the sample:MSTFA ratio on response variation for each of the target compounds in three matrices spiked with standards. Data are presented as relative percentage between responses resulting from 2:3 versus 2:1 ratio.


A
Total area counts
2500000
2000000
1500000
1000000
500000
0
0.5
1
3
5
Minutes
70 ºC
100 ºC
150 ºC
*
B
Total area counts
3000000
2500000
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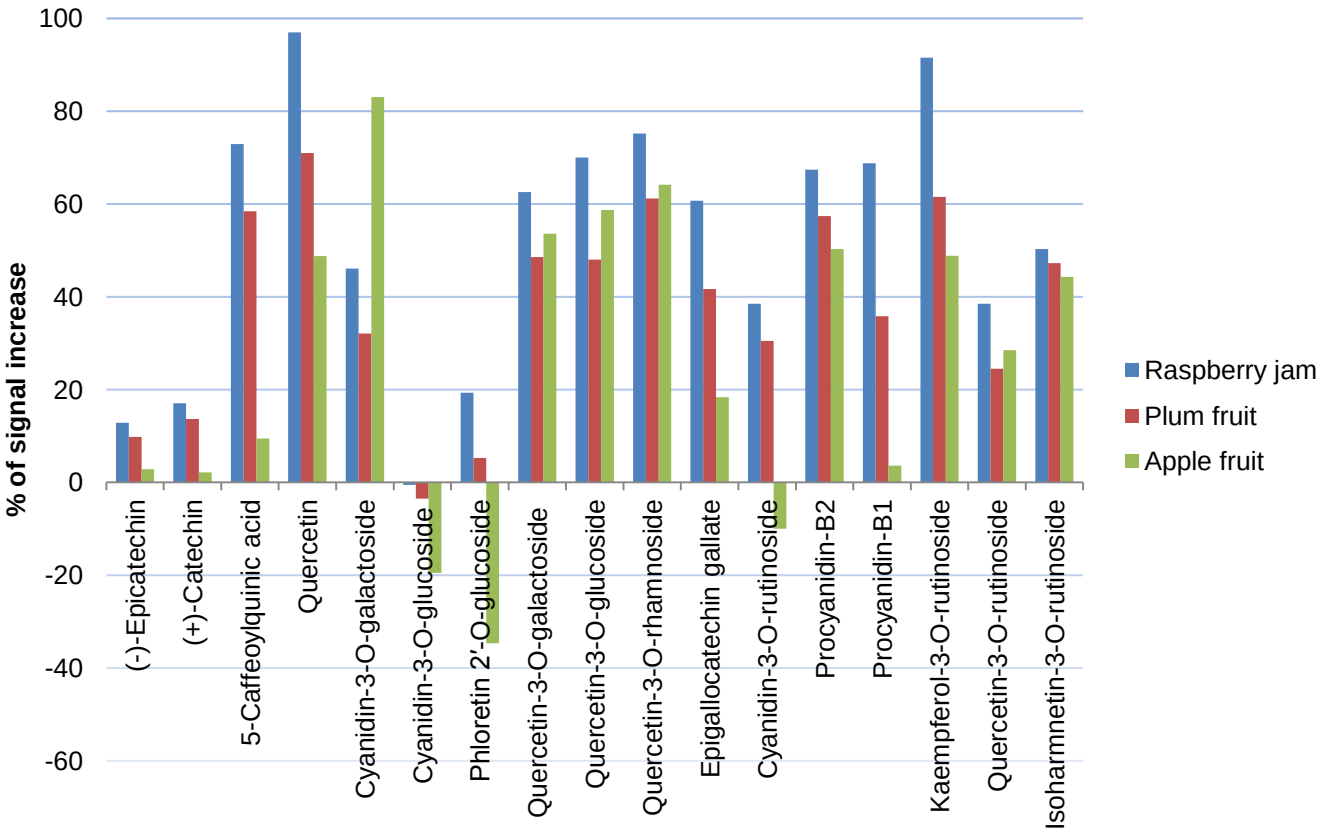
1500000
1000000
500000
0
1/1
1/2
2/1
2/3
3/2
Sample / MSTFA ratio


% of signal increase
100
80
60
40
20
0
-20
-40
-60
(-)-Epicatechin
(+)-Catechin
5-Caffeoylquinic acid
Quercetin
Cyanidin-3-O-galactoside
Cyanidin-3-O-glucoside
Phloretin 2'-O-glucoside
Quercetin-3-O-galactoside
Quercetin-3-O-glucoside
Quercetin-3-O-rhamnoside
Epigallocatechin gallate
Cyanidin-3-O-rutinoside
Procyanidin-B2
Procyanidin-B1
Kaempferol-3-O-rutinoside
Quercetin-3-O-rutinoside
Isoharmnetin-3-O-rutinoside
Raspberry jam
Plum fruit
Apple fruit

- Glycosylated and non-glycosylated polyphenols were analysed by GC-MS/MS.
- Injection port derivatization was optimized in different parameters.
- A C18 SPE clean-up was used to reduce matrix effects.
- The target analysis was applied to several fruit samples.