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Analytical Methods

An improved mass spectrometric method for identification and quantification of phenolic compounds in apple fruits

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ABSTRACT

Thirty-nine phenolic compounds were analysed using ultra high performance liquid chromatography (UHPLC) coupled with diode array and accurate mass spectrometry detection using electrospray ionisation (DAD/ESI-am-MS). Instrumental parameters such as scan speed, resolution, and mass accuracy were optimised to establish accurate mass measurements. The method was fully validated in terms of model deviation ($r^2 > 0.9990$), range (typically 10–3500 ng g^{-1}), intra/inter-day precision (<6% and <8%, respectively) and accuracy (typically 100 ± 10%). The mass accuracy of each selected phenolic compound was below 1.5 ppm. The results confirmed that the UHPLC-DAD/ESI-am-MS method developed here was convenient and reliable for the determination of phenolic compounds in apple extracts.

1. Introduction

Phenolic compounds have received increasing attention in recent years because of their bioactive functions and possible beneficial effects on human health. Epidemiological studies show relations between consumption of polyphenol-rich foods and prevention of diseases such as cancer, coronary heart disease and osteoporosis (Nováková, Spácil, Seifrtová, Opletal, & Solich, 2010; Sato et al., 2011).

Ignat et al. recently reviewed the qualitative and quantitative analysis of phenolic compounds from fruits and vegetables (Ignat, Volf, & Popa, 2011). Despite the large number of investigations made, the separation and quantification of different phenolic compounds, especially the simultaneous determination of phenolic compounds belonging to several subclasses, remains an analytical challenge (Aldini et al., 2011). Method development is hampered by the wide variety of chemical and related physicochemical properties, great differences in concentration, and the lack of commercially-available standards (Vallverdú -Queralt, Jáuregui, Medina-Remón, Andrés-Lacueva, & Lamuela-Raventós, 2010). The challenge is to develop an analytical method that is applicable on a large scale to separate and identify all phenolic compounds of interest (Abad-García, Berrueta, Garmon-Lobato, Gallo, & Vicente, 2009). High performance liquid chromatographic (HPLC) techniques are now widely used for quantification of phenolic compounds (Abad-García et al., 2009). Nevertheless, due to sensitivity disadvantages resulting sometimes in too high detection limits, HPLC methods present limitations for the analysis of complex matrices such as crude plant extracts (Kartsova & Alekseeva, 2008).

These disadvantages make it necessary to perform an initial pre-concentration and purification step to remove potential interfering components prior to HPLC analysis (Ignat et al., 2011).

Applicability calls for a compromise between speed and resolution, resulting in typical analysis times of 45 min or longer (Nováková et al., 2010; Spácil, Nováková, & Olich, 2008). These short comings can be dealt with by using state-of-the-art instruments such as ultra high performance liquid chromatography (UHPLC) systems (Spácil, Nováková, & Solich, 2010). UHPLC allows a higher separation efficiency on sub-2-μm particle sorbents and faster chromatographic separation while keeping the same resolution as HPLC sorbents with a conventional particle size (Guillarme, Nguyen, Rudaz, & Veuthey, 2007). This allows separation and detection of all the phenolic compounds in a single extract from plant material (Gómez-Romero, Segura-Carretero, & Fernández-Gutiérrez, 2010; Lin & Harnly, 2007).

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UV/VIS diode array (DAD) and/or mass spectrometry are the most common detection methods for phenolic compounds, but they share some weaknesses (Harnly, Bhagwat, & Lin, 2007; Spácil et al., 2010). They both lack of structural confirmation and specificity which could lead to possible sample matrix interference and misinterpretation of unknown compounds (Aldini et al., 2011). To identify the compounds, ion trap, single- and triple quadrupole mass spectrometers with electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) are used (Lin & Harnly, 2007; Magiera, Baranowska, & Kusa, 2012). The main limitation of these technologies is that they can only identify and quantify a predefined list of target compounds. These techniques do not allow to perform a non-targeted screening analysis, thereby identifying unknown compounds present in the sample extract. (Abad-García et al., 2009). Furthermore, only a restricted number of target compounds can be simultaneously screened without loss of sensitivity by the aforementioned detection techniques (Moulard et al., 2011).

To remedy these shortcomings, high-resolution mass spectrometers have recently increased in popularity because they can reveal the so-called accurate mass (am) of the analytes. The most common mass spectrometers of this type are time-of-flight (TOF), Fourier transform ion cyclotron resonance (FT ICR) and the Orbitrap detectors (Moulard et al., 2011). Accurate mass measurement coupled with sufficient resolution makes it possible to restrict the enormous number of possible molecular formulas corresponding with a particular molecular mass (Moulard et al., 2011). The fast elemental formula calculation of detected ions made possible by accurate mass measurement is the first step in the identification of unknown compounds and structure elucidation (Vallverdú-Queralt et al., 2010).

In particular, the single stage Orbitrap (Exactive™, Thermo Fisher Scientific, Bremen, Germany) mass analyser provides high mass resolution, high mass accuracy and good sensitivity. In combination with retrospective analysis, this offers a new screening tool to identify phenolic compounds based on accurate mass and isotopic peak ratios (Makarov, 2000; Moulard et al., 2011). Furthermore, due to the sufficiently high scan rates, Orbitrap mass analysers provide sufficient points across narrow chromatographic peaks. This enables the coupling with UHPLC.

The objective of the current study is to set up and validate an identification and quantification method for phenolic compounds (flavonoids, oligomeric flavonoids and phenolic acids) based on UHPLC-DAD/ESI-am-MS, that has the potential to be used as a generic screening method for phenolic compounds. For method development, the peel of apple fruit was the matrix of choice due to the high content of phenolic compounds from several phenolic subclasses.

2. Materials and methods

2.1. Chemicals and reagents

UHPLC-grade methanol, acetonitrile, and water were purchased from Biosolve (Valkenswaard, The Netherlands). Formic acid, acetic acid, ammonium formate, ammonium acetate and (D-Ala)^2-leucine enkephalin were supplied by Sigma–Aldrich (Bornem, Belgium). Commercially available mixtures to calibrate the mass spectrometer, i.e., MSCAL5–1EA (caffeine, tetrapeptide "Met-Arg-Phe-Ala", Ultramark®) for positive ion mode and MSCAL6–1EA (sodium dodecylsulfate, taurocholic acid sodium salt, Ultramark®) for negative ion mode, were purchased from SUPELCO (Bellefonte, PA, USA).

A mixture of different compounds belonging to 7 flavonoid, 1 proanthocyanidin and 4 phenolic acid subclasses was chosen to develop the method. The following analytical reference standards were purchased from Phytolab (Vestenbergsgreuth, Germany): *flavones*: apigenin, apigenin-7-*O*-glucoside (apigetrin), luteolin, luteolin-7-*O*-glucoside (cynaroside); *flavonols:* isorhamnetin, kaempferol, kaempferol-3-*O*-glucoside (astragalin), quercetin, quercetin-3-*O*-glucoside (isoquercitrin), quercetin-3-*O*-galactoside (hyperin), quercetin-3-*O*-rutinoside (rutin), quercetin-3-*O*-arabinoside (avicularin), quercetin-3-*O*-rhamnoside (quercitrin), galangin; *dihydrochalcones:* phloretin, phloretin-*O*-2′-glucoside (phloridzin); *flavanones*: naringenin, naringenin-7-*O*-neohesperidoside (naringin); *flavanols:* (+)-catechin, (−)-epicatechin; *flavanonols:* (+)-dihydrokaempferol ((+)-aromadendrin), (+)-dihydroquercetin ((+)-taxifolin); *anthocyanidins*: cyanidin chloride, cyanidin-3-*O*-glucoside chloride (kuromanin chloride), cyanidin-3-*O*-galactoside chloride (ideain chloride), cyanidin-3-*O*-rutinoside chloride (keracyanin chloride) and *procyanidins*: procyanidin B2. Analytical reference standards of *hydroxybenzoic acids*: salicylic acid, protocatechuic acid, gallic acid, propyl gallate; *hydroxycinnamic acids: p*-coumaric acid, caffeic acid, ferulic acid, sinapinic acid, chlorogenic acid; *hydroxyphenylpropanoic acids*: dihydrocaffeic acid, dihydroferulic acid; *hydroxyphenylacetic acids*: 4-*p*-hydroxyphenyl acetic acid were obtained from Sigma–Aldrich (Bornem, Belgium).

2.2. Preperation of the stock and calibration solutions

Standard stock solutions at a concentration of 1 mg mL^{-1} were prepared in UHPLC-grade methanol for each analyte separately. From the stock solutions, a multi-compound standard solution was prepared in which each of the 39 individual components were present in a concentration of 25 000 ng mL^{-1}. Twenty-three calibration solutions in a concentration range of 25 000–1 ng mL^{-1} were made from the multi-compound stock solution. All solutions were stored at 4 °C in septum-capped amber-coloured vials to protect the compounds from light and moisture. Prior to analysis, each calibration solution was diluted 6:10 (*v*/*v*) in a microvial using a 40 mM ammonium formate buffer, resulting in a calibration series ranging from 15 000 to 0.6 ng mL^{-1}.

2.3. Sample preparation

Apples (Malus × domestica Barkh cv. Kanzi®) were collected during the commercial harvest on September 17th, 2010, at the experimental agricultural station PCFruit (Velm, Belgium). Apples were cooled and stored at 2 °C prior to sample preparation. Prior to freeze-drying, the apples were peeled using a semi-automatic device for a reproducible peel thickness of 3 mm. The samples were immediately frozen in liquid nitrogen to avoid enzymatic browning. Directly from the liquid nitrogen, the samples were transferred into a freeze dryer with heated shelves at 25 °C (GAMMA 1–16 LSC Martin Christ, Osterode am Harz, Germany). Following the freeze-drying process, the apple peel was grounded in a commercial blender (DP705 LA Moulinette, Group SEB, Fleurus, Belgium) and consequently stored under N_2 atmosphere in an amber-coloured flask at −25 °C. The inert atmosphere of N_2 gas avoided rehydration, biological contamination, and compound degradation.

2.4. Instrumental method

Identification and quantification of the selected phenolic compounds were performed via an UHPLC-DAD/ESI-am-MS configuration. The LC system consisted of an Accela™ quaternary solvent manager, a 'Hot Pocket' column oven (Thermo Fisher Scientific, Bremen, Germany) and a CTC PAL™ autosampler (CTC Analytics, Zwingen, Switzerland). A reversed phase separation was performed on a Waters Acquity UPLC® BEH SHIELD RP18 column, with