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HPLC-coupled post-column derivatization aims at characterization and monitoring of plant phytocomplexes, not at assessing their biological properties

Two recent decades of research identified a number of environmental factors as well as mechanisms leading to pathogenic processes in the human organism. A common factor in pathogenesis of many diseases is oxidative stress, a condition in which the production of damaging reactive oxygen species (ROS), present in the body as an inevitable consequence of respiration and exacerbated by the inflammatory response and by exogenous factors such as environmental pollutants, exceeds the capacity of the body's antioxidant defenses to neutralize them. Oxidative damage to DNA can lead to mutations and is therefore a potential precursor of cancer (Hussain et al., 2003). Oxidized lipids (particularly low-density lipoproteins, LDLs) are precursors of plaques that form in blood vessels and result in cardiovascular diseases (Mair, 1997). Oxidative stress is believed to participate in the pathogenesis of atherosclerosis (Park and Oh, 2011) and diabetes (Wang and Hai, 2011). ROS occurrence might also be caused by a disease, e.g. beta-amyloid aggregation in Alzheimer disease, which induces hydrogen peroxide formation (Yin et al., 2011); it may be associated with treatment, e.g. radio- and chemotherapy of cancer (Floyd et al., 2005; Schimmel et al., 2004), and is thought to be a factor in ageing (Sohal et al., 2002). As a consequence, it has been proposed that antioxidants are responsible for the protective nature of foods such as fruits and vegetables, and supplements containing antioxidants are taken daily by millions of people.

In response to this demand, a number of simple analytical methods have been developed to measure total antioxidative activity in various matrices including human samples, vegetables and fruits (raw or processed), beverages, etc. Some of these methods are spectrophotometric or fluorimetric batch tests (Huang et al., 2005; Moon and Shibamoto, 2009); others employ chromatographic resolution of an oxidized (unreacted) or reduced form (after reaction with an antioxidant) of a model reactant following its exposure to a studied sample (Linxiang et al., 2004; Głód et al., 2011). Five such tests have gained the greatest popularity: ABTS and DPPH tests based on bleaching of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) or 2,2-di(4-ter-toctylphenyl)-1-picrylhydrazyl radicals, respectively; the test using Folin–Ciocalteu's reagent measuring active hydroxyl groups, often erroneously suggested as a method for the determination of total phenols; ORAC assay, which monitors the inhibition of oxidative degradation of a fluorescent probe by free radicals generated by azo-initiator compounds; and FRAP assay based on the follow-up of reduction of ferric to ferrous ions at low pH, followed by conversion to a colored ferrous-tripyridyltriazine complex (Huang et al., 2005; Moon and Shibamoto, 2009). It must be stressed, though, that all batch methods employ radicals/oxidants with no physiological relevance, which is their major limitation. Nonetheless, they have a number of advantages, being inexpensive, quick and easy to perform, and in many situations, such as in food production, they are sufficient to compare antioxidant potentials of different samples. Moreover, while it is clear from in vitro and in vivo experiments that antioxidative phytochemicals can reduce oxidative damage, two recent meta-analyses of human cohort and case-control studies with vitamin E (Miller et al., 2005) and with a range of micronutrient supplements (Bjelakovic et al., 2007) concluded that low doses of antioxidants have no significant effect, and high doses might actually increase mortality and disease incidence. Therefore, at this time determination of antioxidant activity should be perceived as a convenient way of detecting reducing compounds in plant and food samples rather than a means of evaluation of their health-promoting potential.

Nonetheless, a number of reducing phytochemicals are indeed valuable bioactive compounds, so their monitoring along the food or dietary supplement production chain is increasingly recognized as an important issue, especially in the case of the so-called functional food industry. Under such conditions, however, the batch tests are insufficient, as they do not allow following up individual components, which is often required. In 1996, a possibility of chromatographic profiling of antioxidants without the necessity of their chemical identification emerged. Since then, papers exploring the principles of DPPH and ABTS tests, later followed by Folin–Ciocalteu's (F–C) assays for on-line, post-column coupling with high performance liquid chromatography (HPLC) have been regularly appearing (Bartasiute et al., 2007; Exarchou et al., 2006; Kool et al., 2007; Kusznierewicz et al., 2011a,b; Milasiene et al., 2007; Niederlander et al., 2008; Shi et al., 2009). The on-line methods aim not only at the rapid measurement of antioxidative activity, but also at the profiling of individual reducing compounds in complex mixtures following their chromatographic separation from the matrix. Detection of antioxidants using post-column derivatization methods is under constant development by a number of researchers (including Irina I. Koleva, Harm A.G. Niederlander, Teris A. van Beek from Phytochemical Section, Laboratory of Organic Chemistry, Wageningen University, and Department of Analytical Chemistry and Toxicology, University Centre for Pharmacy, The Netherlands; Donata Bandoniene, Michael Murkovic from Department of Food Technology, Kaunas University of Technology, Lithuania and Institute of Food Chemistry and Technology, Graz University of Technology, Austria; Angélique Stalmach, William Mullen, Chifumi Nagai, Alan Crozier from Plant Products and Human Nutrition Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Scotland, UK and Hawaii Agriculture Research Center, Hawaii USA, Jules Beekwilder, Harry Onker, Patrick Meesters, Robert D. Hall, Ingrid M. van der Meer, C.H. Ric de Vos from Plant Research International, Wageningen, The Netherlands and PCF-Proeftuin Aardbeien en Houtig Kleinfruit,

Table 1
Antioxidant activity determined for fruit samples using HPLC-coupled post-column derivatization system or standard batch colorimetric ABTS test.

Plant	Type of sample	Method of TEAC determination		Reference
		Batch	Post-column	
Blackthorn	Methanolic extracts [μmol/mL]	54.4	7.7	Kusznierewicz et al. (2011a)
Mirabelle plum		5.9	3.1	
Blue-berried honeysuckle	Lyophilisates [μmol/g d.w.]	246.6	245.9	Kusznierewicz et al. (2012)
Wild growing bilberry		286.2	284.6	
Bog bilberry		274.7	258.6	

Sint-Truidersteenweg Tongeren, Belgium; Zong-Quan Ou, David M. Schmierer, Thomas Rades, Lesley Larsen and Arlene McDowell from School of Pharmacy, Department of Chemistry, University of Otago, Dunedin, New Zealand and Faculty of Health and Medical Sciences, Department of Pharmacy, University of Copenhagen, Denmark, and others), where different methodological conditions have been scrutinized. The most recent addition to this already established method proposed by our group relies on replacing custom-made devices with commercial equipment (Kusznierewicz et al., 2011a,b). The purpose-built prototype devices, usually consisting of reaction coils constructed from PEEK tubing fed by an additional syringe pump, have the advantage of being flexible and customizable according to needs. This is especially beneficial when the duration of the reaction between the antioxidants and the derivatization reagents has to be modified because the kinetics of the reaction might differ depending on reactants. On the other hand, these devices do not ensure interlaboratory compatibility. In contrast, commercial devices require that the chromatographic and derivatization reaction conditions strictly obey the capabilities of the equipment. Once met, though, routine, reproducible analyses can be performed, which is a prerequisite for e.g. monitoring of industrial processes.

Therefore, before we decided to propose an analytical procedure involving a commercial post-column derivatization instrument that could fulfill the requirements of routine protocols, we verified a range of parameters including pH, flow rate, concentration and proportion of the derivatization reagents fed to the effluent from the chromatographic column, etc., as all these factors might influence the measurements (Kusznierewicz et al., 2011b). The most difficult to establish were the conditions ensuring the completeness of the derivatization reaction. As the recommended range of flow rates and pressures in the reaction coil determined the duration of the reaction (about 1 min), the only modifiable parameter was temperature, which determines the reaction kinetics, and consequently the completion of derivatization process prerequisite for reproducibility of results. It is well known that many phytochemicals are thermally unstable. For example, our earlier studies showed that heating chokeberry juice for 15 min at 100 °C resulted in degradation of about 30% of anthocyanins and less than 5% of chlorogenic acids (Kusznierewicz et al., 2011c). Thus, detailed verification of the relationship between temperature in the reaction coil and the outcome of the derivatization process for different reagents (ABTS, DPPH, F-C) was necessary. Experiments carried out for 21 model compounds confirmed that exposure to 130 °C (the maximum temperature achievable in the equipment tested) for less than 1 min caused insignificant or undetectable loss of the phytochemicals tested under conditions enabling proper chromatographic resolution and efficient derivatization of a wide range of structurally different antioxidants (Kusznierewicz et al., 2011a).

Over the past three years, the instrumental setup proposed by our group, consisting of an HPLC, a DAD detector, a commercial post-column derivatizer, a UV–vis detector and an analytical procedure involving gradient elution and post-column derivatization with ABTS radical or F–C reagent at 130 °C have been successfully applied for the profiling of antioxidants in a number of plant samples: mirabelle plum and blackthorn (Kusznierewicz et al., 2011a), blue-berried honeysuckle, bilberry and bog berry (Kusznierewicz et al., 2012), chokeberry (Piasek et al., 2011), rowanberry (Kusznierewicz et al., 2013), coffee beans and brews (Pilipczuk et al., 2011), raspberry, green tea and a dietary supplement (Pickering Laboratories, 2012), as well as medicine plant *Gentiana asclepiadea* (Hudecová et al., 2012) and herbals *Salvia officinalis* and *Thymus vulgaris* (Kozics et al., 2013).

The main superiority of batch determinations of antioxidant activity over profiling of antioxidants, apart from the ease and low cost of measurements, consists in the consideration of synergistic/antagonistic interactions between redox-active components in the sample under study. However, to notice the occurrence of such interactions, it is in fact necessary to use profiling as a reference. This point is illustrated by our comparisons between total TEAC values determined from ABTS batch determination with the sum of TEAC values calculated using areas of peaks detected by post-column derivatization with this radical. As shown in Table 1, among the berry fruits tested, such synergistic interactions between antioxidants increased markedly the determined antioxidant potential only in the case of blackthorn. For the other plant samples investigated, both methods gave similar TEAC values.

As suggested above, the most important current application of methods measuring antioxidant activity is their use for the characterization of plant samples, as well as for monitoring transformations and/or preservation of reducing phytochemicals during processing (although there are no theoretical restrictions against applying these methods for the detection of synthetic redox-active compounds). The possibilities offered by routine, reproducible and reliable profiling of antioxidant components with the aid of HPLC-coupled post-column derivatization are difficult to overestimate. The results of our study in which changes in phytochemical composition of blue-berried honeysuckle juice were monitored upon pasteurization (20 min, 90 °C) demonstrate the benefits of such an approach. As can be seen in Fig. 1, the total antioxidant activity of this juice before and after thermal processing increased when determined by the ABTS assay, and remained essentially unchanged in two other tests used (DPPH and

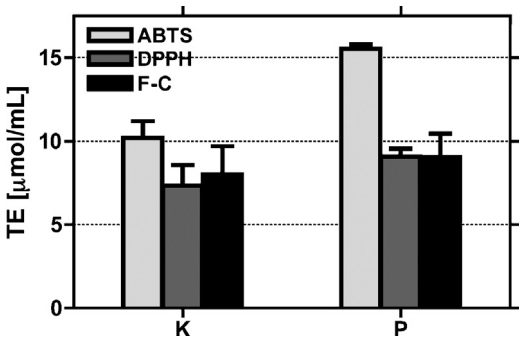

Fig. 1. Total antioxidant activity of fresh (K) and pasteurized (P) blue-berried honeysuckle juices determined by spectrophotometric tests.

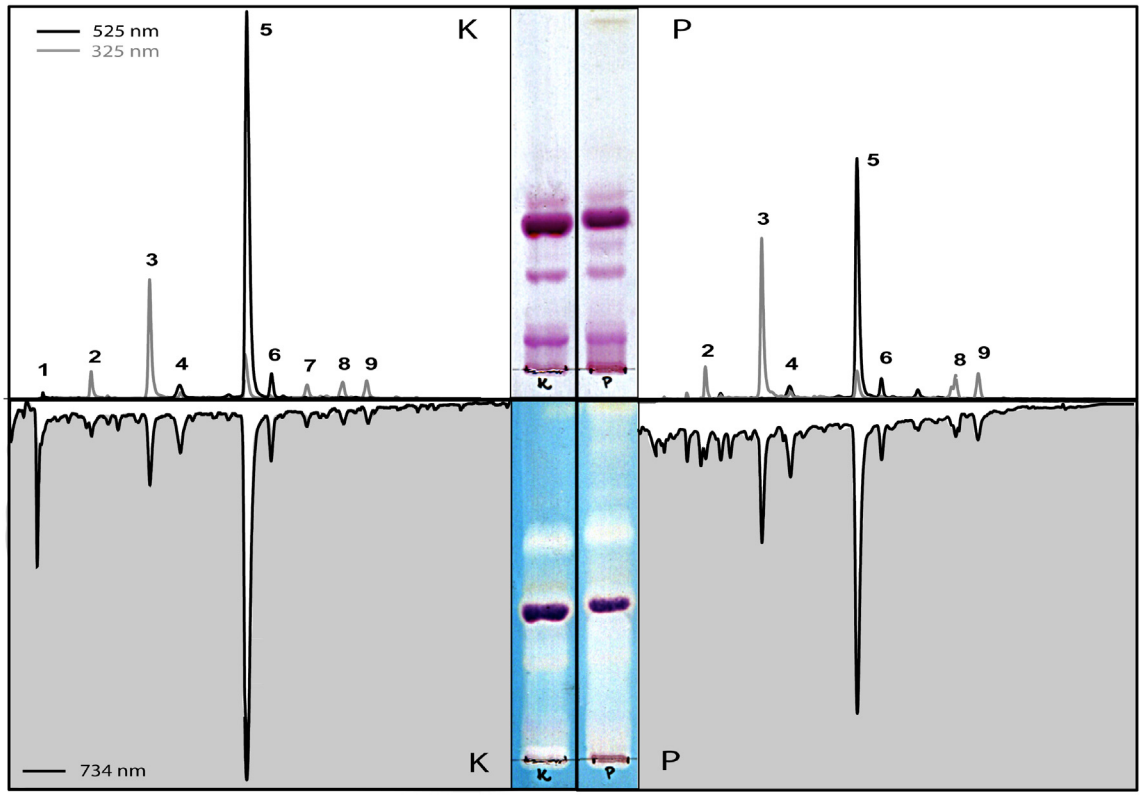

Fig. 2. Combined HPLC and TLC profiles of blue-berried honeysuckle fresh (K) and pasteurized (P) juices, obtained before (upper panels) and after derivatization with ABTS radical (bottom panels). Numbers of peaks correspond to: **1**, vitamin C; **2**, chlorogenic acid; **3**, neochlorogenic acid; **4**, cyanidin-3,5-diglucoside; **5**, cyanidin-3-glucoside; **6**, cyanidin-3-rutinoside; **7**, peonidin-3-glucoside; **8**, quercetin-3-galactoside; **9**, quercetin-3-rutinoside.

F–C). Antioxidant profiling by HPLC coupled with post-column derivatization with ABTS radical provided a number of details with regard to the fate of the juice components during processing.

Significant degradation of vitamin C and lesser, albeit also significant decline of anthocyanins in juices before and after pasteurization reflected by the reduced peak areas obtained by both detectors are clearly seen in the chromatograms presented in Fig. 2. At the same time, increased signals were obtained for products known to be released after anthocyanin degradation: chlorogenic acids (peaks no. 2 and 3) and quercetin derivatives (peaks no. 8 and 9). Moreover, an unresolved band of unidentified ABTS-reactive products formed upon pasteurization appeared both in the chromatogram detected by HPLC-coupled post-column derivatization, as well as in TLC chromatogram visualized with this radical. Thus, despite the loss of some important antioxidant phytochemicals (here vitamin C and anthocyanins), the antioxidative potential of the sample determined by batch methods might remain unchanged providing false information with regard to the influence of processing on sample composition. In the presented example, the degradation of some beneficial compounds was in a way compensated by the formation of other health promoting compounds (phenolic acids and flavonoids); however, the biological activity of the heat-treated sample most probably differed from that of unprocessed juice.

We believe that the data presented provide convincing arguments which should encourage a much broader application of the HPLC-coupled post-column derivatization approach not only in research laboratories, but also in all industry branches involved in processing plant material with the aim to manufacture health-quality food or pharmaceutical products. It cannot be excluded that discrepancies observed between experimentally demonstrated biological activity of purified antioxidant phytochemicals and the lack of confirmed physiological benefits of their consumption in human studies might at least to some extent stem from insufficiently recognized differences between the composition of raw materials and that of the ingested foods. Future research armed with new analytical tools should help close the gap in the understanding of the relationship between chemical make-up and biological properties of plant-based dietary components.

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