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Online coupling of pressurized liquid extraction, solid-phase extraction and high-performance liquid chromatography for automated analysis of proanthocyanidins in malt

Menelaos Papagiannopoulos[a], Benno Zimmermann[a], Annett Mellenthin[a], Martin Krappe[b,1], Giovanni Maio[c,2], Rudolf Galensa[a,*]

[a]*Institute of Food Science and Food Chemistry, University of Bonn, Endenicher Allee 11-13, 53115 Bonn, Germany*
[b]*Abimed GmbH, Raiffeisenstrasse 3, 40764 Langenfeld, Germany*
[c]*Dionex GmbH, Am Wörtzgarten 10, 65510 Idstein, Germany*

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Abstract

A new instrumental setup for automated extraction of solid samples by online coupling of pressurized liquid extraction, automated SPE (solid-phase extraction) and HPLC is presented. From the extraction to the chromatogram no manual sample handling is required. The application to the determination of proanthocyanidins in malt reduces time and manual work to a minimum compared to former manual methods. Twenty samples can be processed within 24 h in respect to eight samples with the manual method. Using the features of the instrumental coupling, an optimized strategy for SPE of proanthocyanidins from natural samples was developed, requiring no evaporation step, using commercial cartridges and delivering concentrated eluates. The recovery of five main malt proanthocyanidins was 97%, with a reproducibility of 5%. This new instrumental coupling is thought to reduce time and costs along with improved results for a broad range of solid sample materials.

Keywords: Pressurized liquid extraction; Solid-phase extraction; Extraction methods; Automation; Instrumentation; Beer; Food analysis; Proanthocyanidins; Polyphenols; Phenols

1. Introduction

Proanthocyanidins are a group of phenolic plant metabolites comprising a large number of similar compounds. They exhibit promising physiological effects [1–3] and are of technological importance due to their antioxidant activity [3–6] and protein binding potential. In beer, proanthocyanidins are related to flavour and foam stability [6–8] and to the formation of haze [7–13].

Complex matrices like samples from natural origin often require laborious sample preparation (prepurification and preconcentration) prior to chromatographic analysis. For the extraction of proanthocyanidins from barley and malt, time consuming manual multistep solid–liquid and liquid–liquid extractions with

*Corresponding author. Tel.: +49-22-873-3798; fax: +49-22-873-3757.
E-mail address: galensa@uni-bonn.de (R. Galensa).
[1]Present address: OmegaTech GmbH, Mikroforum Ring 2, 55234 Wendelsheim, Germany.
[2]Present address: Dionex Softron GmbH, Dornierstrasse 4, 82110 Germering, Germany.

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acetone, methanol, ethyl acetate, water or mixtures are commonly applied, including solvent evaporation steps [14–17]. Often, a purification by column chromatography is used with a broad range of stationary phases, among them commonly polyamide, and methanol, acetone or dimethylformanide (DMF) for elution of proanthocyanidins [8,12,18–21]. Some authors have used solid-phase extraction (SPE) with modified RP-18 adsorbents and acetone–water mixtures for elution [22,23].

The manual work and time required by these methods are not only inconvenient but also include the risk of analyte degradation and formation of artifacts due to the sensitivity of proanthocyanidins to temperature, oxygen and light. Pressurized liquid extraction (PLE; Dionex trade name ASE, for accelerated solvent extraction) uses elevated temperature and pressure for the extraction of solid samples. Compared to manual or Soxhlet extraction, complete extraction can be obtained in a very short time due to enhanced kinetics and better penetration of the sample by the solvent. Despite its rapid acceptance in environmental sciences [24–27], PLE has yet found little use in food analysis, especially in the analysis of polyphenols in food. Up to now, only the application of PLE to the analysis of resveratrol in grape pomace [28] and proanthocyanidins and flavonoids in apple [29,30] has been described. The ASPEC (Automated Sample Preparation with Extraction Cartridges) is a sampler for automated SPE and serves as an autosampler for injection in LC systems [31–33].

In this paper, we present the online coupling of PLE, automated SPE and HPLC (see Fig. 1), which allows to process solid samples from the extraction to the chromatogram automatically. A preliminary description of this novel hardware combination was shown at a meeting of the GDCh (abstract of the oral presentation see Ref. [34]) and at the Eurofoodchem XI [35]. This new setup was applied to the determination of proanthocyanidins in malt from barley. The necessary adaption of the hardware setup and modifications to the formerly used manual method are described and compared. An optimized SPE method with commercial polyamide SPE cartridges is used for the purification of the extracts, that delivers very pure and concentrated eluates.

2. Experimental

2.1. Samples

Malt samples from the barley varieties Prisma and Caminant were kindly provided by Weissheimer Malz (Andernach, Germany) and stored at +4 °C. Malt was ground in portions of 7 g each in a ball mill (MM Retsch, Haan, Germany) at room temperature for 10 min and stored at room temperature in the dark until analysis.

Fig. 1. The hardware setup: coupling of PLE, automated SPE and HPLC (from left to right: ASE 200, ASPEC XLi, HPLC).

Standards of prodelphinidin B3 [gallocatechin-(4α→8)-catechin] and prodelphinidin C2 [gallocatechin-(4α→8)-gallocatechin-(4α→8)-catechin] were a kind gift from F. Petereit, Institute of Pharmaceutical Biology and Phytochemistry, University of Munster, Germany. For details of the nomenclature, see Ref. [18].

2.2. Extraction

The PLE of ground malt was carried out using an ASE 200 system (Dionex, Idstein, Germany). Four grams of ground malt was mixed with 1.8 g of diatomaceous earth (Isolute HM-N, Separtis, Grenzach-Wyhlen, Germany) and filled in 11 ml stainless steel extraction cells. Optimized extraction conditions are shown in Table 1. Solvent composition, temperature, static extraction time and number of cycles were varied to optimize the extraction. The collection vials were lifted by 2 mm (by putting a collection vial septum under the collection vial) to reduce the extract volume to a minimum. For comparison, manual extraction was carried out according to a previously published method [18].

2.3. SPE

Purification of the extract was performed by an ASPEC XLi system (Gilson, marketed by Abimed, Langenfeld, Germany) directly coupled to the ASE 200 for automated transfer of the extracts to the SPE cartridges. Collection vial septa were cross cut for compensation of pressure differences while aspirating the extract from the ASE 200 collection vial.

Table 1
Optimized PLE parameters

Solvent	Acetone–water (80:20, v/v)
Sample amount	4 g
Temperature	60 °C
Pressure	100 MPa
Preheating time	5 min
Static extraction time	10 min
Number of cycles	1
Flush volume	50%
Purge time with N_2	60 s
Time per sample	25 min
Extracting volume	14 ml

The ASPEC system performed the following tasks after completion of the extraction by PLE: The SPE cartridge was conditioned with 7 ml water. In the ASE 200 collection vial, the crude extract was diluted with 24 ml water and mixed in liquid mode by aspirating and dispensing 10 ml of the diluted extract. The extract was loaded onto the SPE cartridge quantitatively, the liquid pushed through the cartridge with air and the cartridge washed with 8 ml water and 1 ml DMF–water (85:15, v/v). Finally, the adsorbed analytes were eluted with 2.5 ml of the latter solvent.

SPE cartridges used were of 6 ml size, packed with 1 g polyamide (Macherey–Nagel, Düren, Germany).

2.4. Coupling of the ASE and ASPEC systems

The two instruments are coupled by the ASE-ASPEC kit, a development of Abimed and Dionex, which is now commercially available.

The ASE 200 is placed at the left side of the ASPEC, so that the needle of the ASPEC has access to the collection vial tray of the ASE (see Fig. 1). The position of the ASPEC needle holder is changed to the left side of the horizontal arm to allow the ASPEC to move its needle above the collection vial.

After completion of the extraction the ASE moves the collection vial to a position accessible by the ASPEC. The extraction sequence is paused for the time needed by the ASPEC. After completion of extract transfer, the next extraction is started. Communication between the two instruments is accomplished by contact-closure.

2.5. HPLC

For HPLC analysis in coupled mode, 20 μl of the SPE eluate were injected by the ASPEC via a Rheodyne six-port valve. The Beckman System Gold HPLC (Beckman Coulter, Unterschleissheim, Germany) consisted of a Degasys 1210 degasser (Uniflows, Tokyo, Japan), a solvent delivery module 126

set to a flow of 1.2 ml/min, a Luna RP-18 column, 150×4.6 mm I.D., particle size 5 μm (Phenomenex, Aschaffenburg, Germany) maintained at 30 °C and a scanning detector module 167 set to 280 nm. The mobile phases were (A) NaH_2PO_4 0.02 *M*, pH 3.4 and (B) acetonitrile–NaH_2PO_4 0.05 *M*, pH 3.0, (2:1). Gradient conditions: After holding the initial 5% B for 1 min, a linear gradient to 20% B during 14 min eluted all analytes of interest. A washing step with 100% B for 5 min and subsequent reequilibration to initial conditions for 10 min concluded the program. The HPLC method is a modification of a previously reported method [18].

For method development and comparison with previously published results [18], selected samples were run on an HPLC system equipped with a coulometric electrode array detector comprising ten electrodes set to 0 to 990 mV in steps of 110 mV (ESA, Chelmsford, MA, USA). All other chromatographic conditions were as described for coupled analysis.

2.6. Mass spectrometry

For structure verification, selected samples were subjected to multiple step mass spectrometry (MS^n) analysis using a Summit HPLC system (Dionex) coupled to an LCQ ion trap mass spectrometer (ThermoFinnigan, Egelsbach, Germany) with an electrospray ionization (ESI) interface. For compatibility reasons, the mobile phases were changed to (A) 2% (v/v) acetic acid solution in water and (B) 2% (v/v) acetic acid solution in acetonitrile, delivered with a flow-rate of 0.2 ml/min. An Aqua RP-18 column, 250×2 mm I.D., particle size 5 μm (Phenomenex) maintained at 30 °C was used and the HPLC eluent mixed with 0.1 ml/min methanol before entering the ESI interface, to enhance ionisation efficiency in negative mode.

MS–MS experiments were conducted on the monomeric, dimeric and trimeric proanthocyanidin pseudomolecular masses in addition to full scan MS. Comparison of the fragment spectra was used to verify the identity of the six prominent peaks A–F (see Fig. 4), as has been described in detail previously [18].

3. Results and discussion

3.1. Extraction

The settings for PLE were optimized (see Table 1) based on the manual extraction method. This method is suitable to extract 99% of the proanthocyanidins. As for the manual method [18], acetone–water (80:20, v/v), was found optimal as extraction solvent. A temperature lower than 60 °C results in inefficient extraction and higher temperature shows analyte loss due to thermal degradation. However, using 60 °C no thermal degradation during the extraction was observed. A pressure setting of 100 or 200 MPa showed no difference. The results regarding solvent, temperature, time and pressure variation are comparable to those found by others [28] for extraction of phenolics by PLE. The total time for a single sample extraction is approximately 25 min compared to 2 h with the manual method.

By PLE, no difference in extraction yield could be seen between malt ground in a ball mill at room temperature or under cooling with liquid nitrogen (resulting in finer powder) as needed for manual extraction [18]. Up to 4 g of ground malt can be loaded into one extraction cell to obtain high concentrations of analytes in the resulting extract. The extract volume can be reduced to approximately 14 ml by fully packing the extraction cells.

3.2. SPE

Due to the amount of interfering matrix compounds and the low concentration of proanthocyanidins a purification and concentration step is required prior to HPLC–UV and –MS analysis.

Previous investigators found it necessary to completely exclude organic solvents from the solution to be loaded onto the column to prevent analyte loss due to impaired retention. Therefore, evaporation is used to reduce the volume and to provide an aqueous solution.

Evaporation of solvents is incompatible with the presented instrumental setup, but extraction volumes are much smaller. By loading malt extracts with different contents of acetone onto polyamide SPE cartridges, an analyte breakthrough of less than 2%

of prodelphinidin C2 [gallocatechin-(4α→8)-gallocatechin-(4α→8)-catechin] and prodelphinidin B3 [gallocatechin-(4α→8)-catechin] could be observed with up to 30% acetone (Fig. 2). Other proanthocyanidins were not detected. Therefore it was feasible to dilute the extract with water to reduce the acetone content to 30% by increasing the total volume to 38 ml.

Taking advantage of the features of the ASPEC, the elution can be optimized easily. Different solvents and solvent mixtures were investigated for optimization of the elution from the SPE cartridges. As illustrated in Fig. 3, DMF–water (85:15, v/v) showed to be the most efficient eluent. Furthermore, the loaded cartridges were eluted in five fractions of 1 ml each. The first milliliter of DMF–water can be discarded, since it doesn't contain any proanthocyanidins, while the second and third milliliter

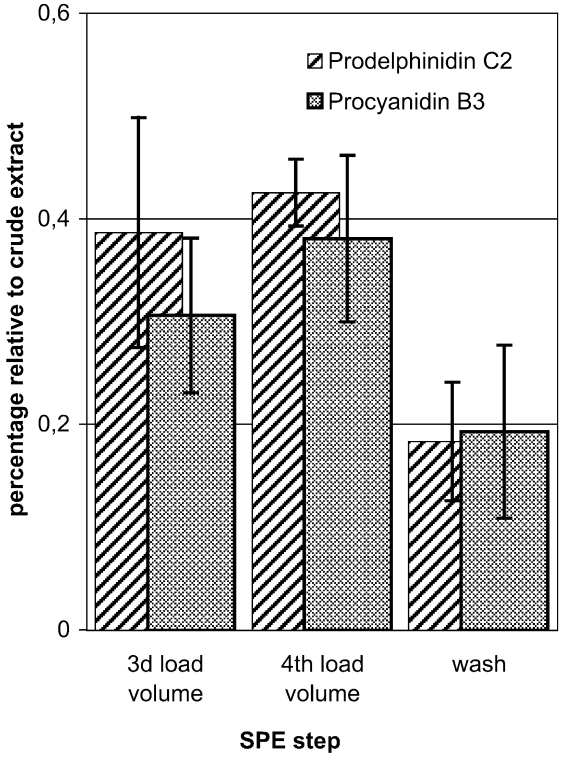

Fig. 2. The diluted crude extract was loaded onto the SPE cartridges in four fractions of 10 ml each and washed with 8 ml water; the slight breakthrough of two analytes is shown (last two load fractions and wash volume).

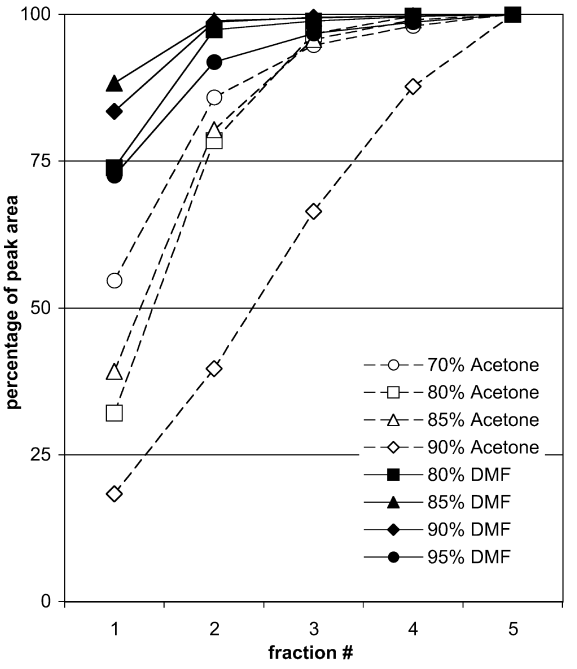

Fig. 3. The cumulated percentage of eluted prodelphinidin B3 from the SPE cartridge by different solvent/water mixtures is shown; using 85% DMF, more than 98% can be eluted in two fractions.

contain more than 98% of the total proanthocyanidins (see Fig. 4).

3.3. HPLC

For HPLC analysis of extracted and purified proanthocyanidins, modifications were made to a previously published gradient method [18]. Using a shorter column with reduced particle size and higher flow-rates, analysis time could be decreased from 71 to 30 min including washing and reequilibration. Thus, the HPLC method is as short as the PLE and the SPE method, enabling efficient coordination of all instruments involved.

The peaks of the proanthocyanidins were identified by comparison of retention time, voltammogram and UV spectrum to previously published data [18]. The identity was further confirmed using LC–MS^n. Fig. 4 shows the UV chromatogram of a SPE eluate from Prisma malt at 280 nm with the identified compounds labelled.

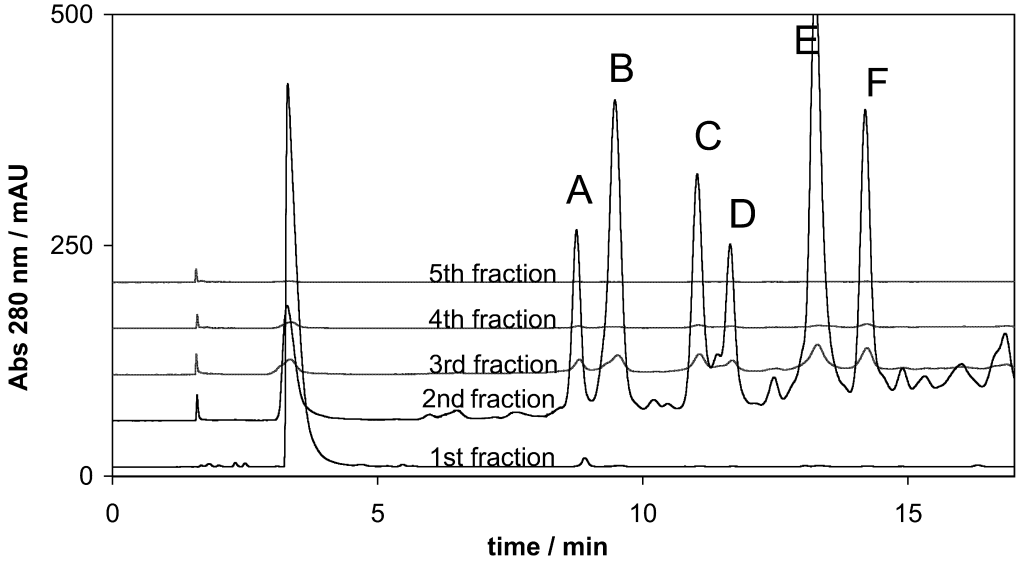

Fig. 4. To optimize the elution volume, the SPE cartridge was eluted in five fractions of 1 ml DMF 85% each. The chromatograms show that the second and third fraction only contain the analytes; identified compounds: A: *prodelphinidin C*2 (*G*–*G*–*C*); B: *prodelphinidin B*3 (*G*–*C*); C: *G*–*C*–*C*; D: *C*–*G*–*C*; E: *procyanidin B*3 (*C*–*C*); F: *C*–*C*–*C* coeluting with *catechin*; (*C* = catechin unit, *G* = gallocatechin unit).

3.4. Recovery, reproducibility and method efficiency

Due to the lack of commercially available standards it is not feasible to determine the recovery and reproducibility of individual compounds by standard addition in all cases. For this work, only catechin, prodelphinidin B3 and prodelphinidin C2 were available as standards. To circumvent this problem, two different approaches were made to determine the reproducibility and the recovery of the new method: a calculation by (1) relative quantification and by (2) standard addition.

1. Relative quantification. The peak area of the peaks A to E (see Fig. 4) was compared on different stages of the analytical procedure. That enables to calculate recovery and reproducibility without the necessity to determine absolute amounts of the compounds. Peak F was not used for these calculations, since it consists of two coeluting compounds (see Fig. 4).

   In this first approach, three batches of six Prisma malt samples each were subjected to the following procedure:

   To determine the recovery and reproducibility of PLE, prisma malt samples were extracted twice by PLE. The amount of each of the five proanthocyanidins in the resulting PLE extracts without further purification was determined.

   The peak area of each of the compounds in the first extract relative to the sum of both extracts was 98–99% for each of the five analytes considered. The reproducibility was 2.5–3.5% for each of the five compounds. Compared to the manual method, there is no significant difference in the extraction yield.

   To determine the recovery and reproducibility of the SPE, malt samples were extracted by PLE and the extracts pooled and aliquoted to provide sample extracts of equal volume and composition. These were subjected to SPE using the ASPEC. The peak area of the five proanthocyanidins in the resulting SPE eluates was compared to those in the crude extract.

   The recovery for SPE was calculated to be better than 98% for each of the five compounds covering losses due to analyte breakthrough, matrix interferences and irreversible adsorption to the polyamide. The reproducibility calculated from the 18 independent determinations was 3%

for each of the compounds. With the manual SPE method, a considerable lower recovery of 87 and 84% for catechin and prodelphinidin B3, respectively, was determined.

2. Standard addition. In this second approach, Caminant malt, known for its low content in proanthocyanidins (see Section 2.1), was spiked with catechin, prodelphinidin B3 and prodelphinidin C2 and the malt sample subjected to PLE and SPE. Accounting for the limited amount of compounds available, this was done in duplicate only. The three compounds were quantified in the stock solution used for standard addition, in the crude PLE extracts and in the SPE eluates and the recovery of the analytes was calculated. There was no significant difference in the findings from standard addition and the results from the relative quantification of the first approach.

Taking into account the two different approaches, the recoveries of the PLE and the SPE alone were 99 and 98%, respectively, for each of the five different compounds. This results in a recovery of 97% for the overall method. The reproducibility of the PLE and the SPE alone was 3 and 3%, respectively, and 5% for the overall method. With the manual method, a recovery of 85% and a reproducibility of 5% can be achieved. A comparison of the relative amounts of the individual compounds in samples prepared manually and with the presented method showed that there is no significant difference in selectivity between the two methods. This ensures the comparability of results obtained previously by the manual sample preparation. The time consumption of the automated method is remarkably lower than that of the manual one. For a single sample, the total analysis time from extraction to the chromatogram is approximately 6 and 1.5 h, respectively (see Fig. 5). Taking into account the parallel processing of samples with manual work, eight samples can be analyzed in one working day (8 h), whereas the automated method can process up to 20 samples in 24 h. Additionally, the shortened analysis time together with the preponderant exclusion of oxygen in the ASE system reduces the risk of analyte breakdown and artifacts due to oxygen, light and temperature.

4. Conclusion

A new instrumental setup for the automated analysis of solid sample materials by online coupling of PLE, automated SPE and HPLC is presented in this paper. Furthermore an advanced SPE method is introduced. The application to the analysis of proanthocyanidins in malt samples demonstrates the benefits of the new method, resulting in reduced analysis time, higher throughput and improved recovery. With the necessary modifications, this approach is thought to be useful for a number of different applications dealing with solid sample materials.

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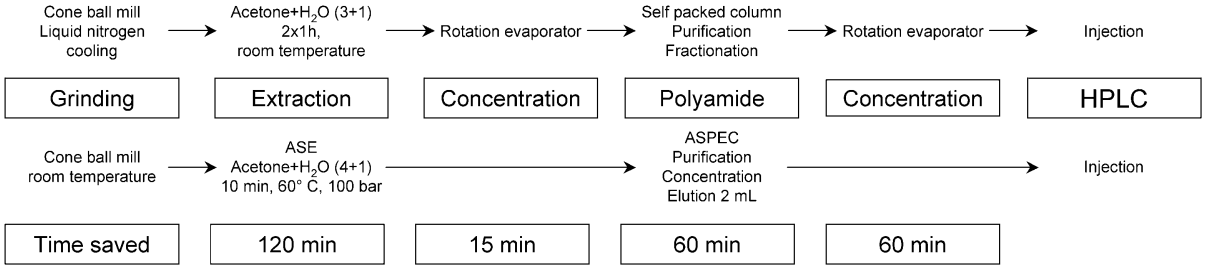

Fig. 5. Comparison of the former manual method (above) to the new automated method (below).

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