FPL 2: Alternatives to Chromatography in Plant Breeding

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Abstract

Wild plants were taken into cultivation because of special features. Usually, medicinal plants or spices show distinct secondary metabolites combined with a specific pattern of these compounds. Typically, chromatographic methods like gas chromatography (GC) or high performance liquid chromatography (HPLC) were applied as standard methods for a meaningful analysis of secondary metabolites. However, these methods are labor and time intensive. In the breeding process, usually numerous single plants have to be analyzed and therefore, high throughput methods are required. In this article, some examples for alternative strategies are given. Besides spectroscopic methods like near infrared (NIR), also biosensoric approaches should be considered. For instance, several enzymes can oxidize or hydrolyze secondary metabolites in dependence of their functional groups. Polyphenols can be determined by laccases. Polyphenols like catechins and flavonoids contribute to the bioactivity of many medicinal plants. Also cysteine sulfoxides, which are typical for *Allium* species like garlic and onions, can be enzymatically determined with high specificity. Finally, toxic cyanogenic glycosides can be quantified by the enzyme cyanidase.

Keywords: biosensors, polyphenols, cysteine sulfoxides, cyanogenic glycosides

Introduction

Selection processes are required for all types of breeding approaches. In a more classing setting, characters like growth or environmental resistance are used as selection criteria. In the case of vegetable plants, nutrients like carbon hydrates, fat or protein have to be analyzed. In terms of medicinal plants or spices, specific secondary metabolites have to be determined in a qualitative and quantitative manner.

This situation is rather clear for essential oil plants. The content of essential oil can be determined by standard methods like steam distillation and the pattern of individual compounds is usually analyzed by gas chromatography (GC). For all other compounds, analysis is more complicated; usually high performance liquid chromatography (HPLC) is used for this task. However, chromatographic conditions have to be adapted to each group of secondary metabolites as well as each plant species. During the breeding process, typically several hundreds of samples have to be analyzed in a rather narrow time frame and therefore, high throughput methods are required. However, chromatography is less suitable for such a high throughput approach.

Therefore, alternative strategies have to be worked out. Rather powerful are spectroscopic methods like near infrared spectroscopy (NIR). There are several examples that especially for essential oil plants a result can be archived within several minutes (SCHULZ AND BARANSKA, 2007). Additionally, Raman infrared spectroscopy in combination with microscopy allows identification of certain compounds in plant tissue. For instance, the distribution of rather unique thiopyrrols in the stems of the wild *Allium* species *A. jesdianum* BOISS. et BUHSE can be visualized (Fig. 1). These compounds are typical for wild onions in Middle Asia, which are highly estimated as vegetable and medicinal plant.
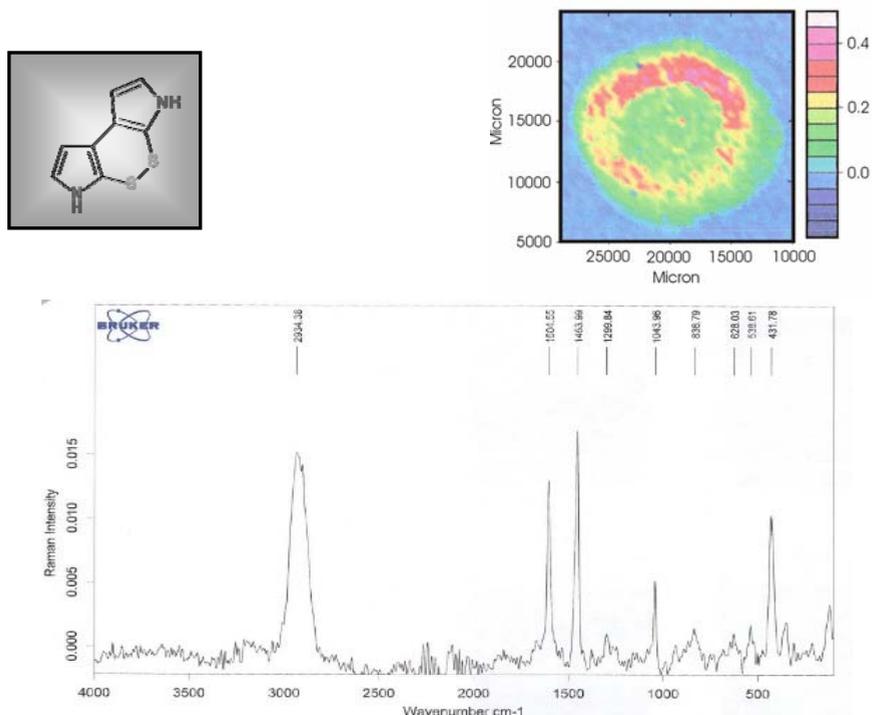
Fig. 1 Localization of thiopyrrols in stems of Allium jesdianum by IR-Raman-microscopy (scan on sharp and distinct peak at 1453 cm-1). The thiopyrrol seems to be located in the tissue surrounding vessels. On the left side box, the suggested structure of the thiopyrrol and the corresponding Raman IR is depicted below. Measurements were kindly provided by Prof. H. Schulz, JKI Quedlinburg.

Besides spectroscopic methods, also further alternative approaches should be considered. For instance, larger molecules can be recognized by specific antibodies. As a disadvantage, specific antibodies have to be designed firstly in a work intensive process. Also, antibodies show some cross specificity. As a further alternative, enzyme-based approaches are possible. Usually, degrading enzymes do not specifically act on individual secondary metabolites, but can be used for group determination. Most suitable are hydrolases and oxidoreductases. Hydrolases cause a slight pH shift of the sample solution, which can be detected by pH-sensitive devices, whereas oxidoreductases can be combined with amperometric sensors.

Combination of these enzymes with potentiometric electrodes, *e.g.*, pH electrodes or amperometric electrodes, can be utilized for the construction of biosensors (KEUSGEN, 2002). A biosensor is defined as a tight combination of a biological recognition element like enzymes or antibodies with a physical transducer like electrodes. The biological element is immobilized on the surface of the physical transducer (BARLEN et al., 2009); such a biosensor is reusable. With this type of sensors, several hundreds of measurements can be performed within one day. The sensor can be also miniaturized. In this article, three different approaches are described, which are suitable for plant breeding approaches as well as quality control of herbal products.

Materials and Methods

Key elements of the here presented biosensoric applications are the enzymes as mentioned in Fig. 2. Alliinase has been obtained from fresh or powdered garlic (*Allium sativum* L.; KREST AND KEUSGEN, 1999). Bacterial cyanidase has been used as recombinant enzyme (KEUSGEN et al., 2004). Laccases from *Trametes versicolor, Agaricus bisporus* and *Rhus vernificera* were obtained from Sigma-Aldrich.

Electrodes like depicted in Fig. 3 were bought from BVT Technologies (Brno, Czech Republic). Best results were archived with type AC1.W2.R1 (platinum working electrode). A mobile PalmSens potentiostat (Palm Instruments BV, Houten, Netherlands) at a potential of E=670 mV was used for measurements as shown in Fig. 4 and Fig. 5. Further details are given in Schmidt (2012).

For immobilization of the laccase, the platinum working electrode of the sensor as shown in Fig. 3 was treated four times with 0.25 µL of a 4-fluoro-3-nitrophenyl azide -(FNPA)-solution (10 mg/mL; good solubility in *tert*-butyl methyl ether) and irradiated for 1 h with UV light at λ=254 nm. Directly after irradiation, 0.5 µL of the laccase solution (35 mg/mL) was added and the sensor was dried at room temperature. The senor was rinsed with H_2O and stored in phosphate buffer (pH 7.0, 0.1 M) preserved with Thiomersal (0.05 %) before further use at +4 °C.

As a control, total phenol content has been determined according to Folin-Ciocalteu (Singelton and Rossi, 1965). Samples with a high content of phenolic compounds (> 1g/l) have to be diluted before measurement. From this aqueous test solution, 0.1 mL was taken and diluted by 8.4 mL of H_2O followed by 0.5 mL of the Folin-Ciocalteu reagent. After 5 min, 1.0 mL 0f saturated sodium carbonate solution was added and the absorption at λ=720 nm has been measured. For calibration, (-)-epicatechin was used; therefore all concentrations given in Fig. 4 and Fig. 5 are expressed in (-)-epictechin equivalents. For further details see Schmidt, 2012.

All real samples were obtained from local stores. Aronia and Cranberry juice could be directly used after filtration and dilution, whereas the samples of green tea were prepared as infusion. 12 g of tea leafs were given into 1 L of 90 °C hot, demineralized H_2O. After 5 min, the infusion has been filtrated and stored at +4 °C before measurement. Shortly before measurement, the sample has to be diluted in an appropriate manner (typically phosphate buffer pH 7.0). It has to be considered that phenols are easily oxidized by air oxygen and therefore samples should be tightly closed (best under inert gas atmosphere) as well as measurements should be performed as soon as possible after sample preparation.

Results and Discussion

Three different enzymes were tested as part of biosensor electrodes (Fig. 2). In all cases, the sensors were applied to chemical standards and to real plant samples. In Fig 2a, the so called 'laccase reaction' is shown. This enzyme is widely distributed in nature, especially in fungi like *Trametes versicolor* and *Agaricus bisporus* as well as in higher plants like *Rhus vernificera*. All three enzymes were tested with different standards and the laccase from *T. versicolor* was found to be most suitable for a biosensoric approach. The enzyme has been immobilized on the working electrode of a thick film electrode (TFE, Fig. 3) by different methods. Best results were archived by using the photo cross-linker 4-fluoro-3-nitrophenyl azide (FNPA). Alternatively, the enzyme can be immobilized on carbon nanotubes, entrapped into polylactic acid (PLA) or immobilized by covalent immobilization via amide binding. All these strategies are possible, but not superior over photo cross-linking by FNPA (Schmidt, 2012).

Laccase is a rather unspecific enzyme. Its function in nature is formation of radicals (Fig. 2a) followed by cross-linking like the formation of lignans. If polyphenols were used as educts, enzymatic products are typically of brown or black color; this reaction naturally occurs after tissue wounding in is easily visible by discoloration within seconds. A similar reaction can be catalyzed by phenoloxidases (Schmidt, 2012). Because of the low substrate specificity of laccase, a wide range of phenolic compounds can be determined by a biosensor based on this enzyme.

The obtained sensor has been calibrated with (-)-epicatechin in a concentration range between 10 and 100 µg/mL. The detection limit was determined to be at 7.7 µg epicatechin per mL. The pH of the test solution was adjusted at pH 7.0. In the used experimental set up, measuring time for a single sample was 5 min. In the next step, different real samples were analyzed and obtained results were compared with a standard reference method (Folin-Ciocalteu phenol determination). As shown in Fig. 4, the biosensor and the reference values gave a good correlation, but the bio-

sensor values are systematically about 20 % higher as results gained by the Folin-Ciocalteu method. There is no explanation for this phenomenon. However, it must be considered that the investigated samples contain complex mixtures of polyphenols, which are mainly catechin derivatives.

Fig. 2 Three different enzymatic reactions, which can be used for biosensor approaches. A) laccase reaction for the determination of phenolic compounds, b) alliinase reaction for the determination of cysteine sulfoxides and c) cyanidase reaction for the determination of cyanogenic compounds.
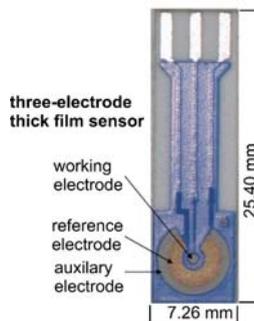
Fig. 3 Photo of a commercial thick film electrode (TFE; Schmidt, 2012). The enzyme has to be immobilized on top of the platinum working electrode.

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As depicted in Figure 5, two different types of juices have been investigated. Both juices showed a phenol content which is much higher as for the green tea. This requires a 100fold dilution of the samples before measurement. Surprisingly, the found amount of phenolic compounds for Aronia juices was 50 % lower as determined by the reference method. An explanation can be that Aronia is also rich in Vitamin C, which is also highly redox-active and can therefore interact with measurements by chemical reduction of formed radicals. This phenomena might lead to decreased polyphenol values by the here presented electrochemical method.

In the case of Cranberry juice, results of both methods were nearly identically. It has also to be considered that the calibration was performed with epicatechin, which is typical for tea but of less importance for fruit juices. Phenolic compounds in plants occur normally as a mixture of several compounds and therefore, calibration with only one compound is somewhat problematic. But nevertheless, the here presented method is well suitable for relative comparison of samples of a distinct plant species.

It also must be pointed out that results are more precise if the biosensor is placed inside a flow-through-analyzer (FIA). Such a FIA-approached has been published by KEUSGEN in 1998. However, the miniaturized sensor as depicted in Fig. 3 can be also used in the field because the used PalmSens potentiostat can be operated by batteries. Fresh plants can be analyzed by giving one drop of juice on the TFE sensor surface, but these measurements are less precise as using the FIA device.

Further on, some other secondary metabolites can be determined by enzymatic biosensors. As depicted in Fig. 2b, cysteine sulfoxides, which are typical for all onions, can be digested by the

enzyme alliinase. In this case, the alliinase was obtained from garlic. This reaction is rather interesting because it results in a slight pH-shift of the sample solution which can be detected by ph-sensitive semiconductor devices (KEUSGEN et al., 2003). Alternatively, the formed ammonia can be quantified by an ammonia electrode or a comparable method (KEUSGEN, 1998, MILKA et al., 1999). As a second alternative, the pyruvate might be enzymatically determined by conversion with lactate dehydrogenase into lactate (LDH; KREST AND KEUSGEN, 1999). The lactate formation correlates directly to the pungency of garlic and onions. The biosensoric method based on ammonia quantification could be successfully applied to several samples of garlic and wild onions (MILKA et al., 1999, KREST AND KEUSGEN, 2002, KEUSGEN et al., 2003). By using an automated FIA device, a single analysis needs 3 min.
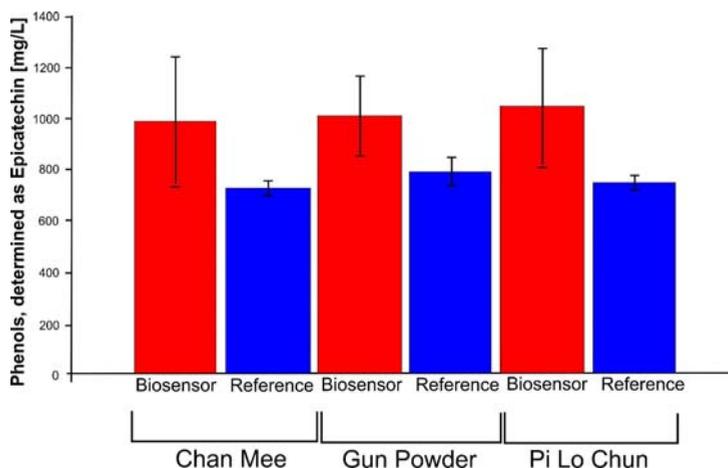
Fig. 4 Results of green tea samples (12 g dried leafs in one L of hot water). Reference values were obtained by the Folin-Ciocalteu method.
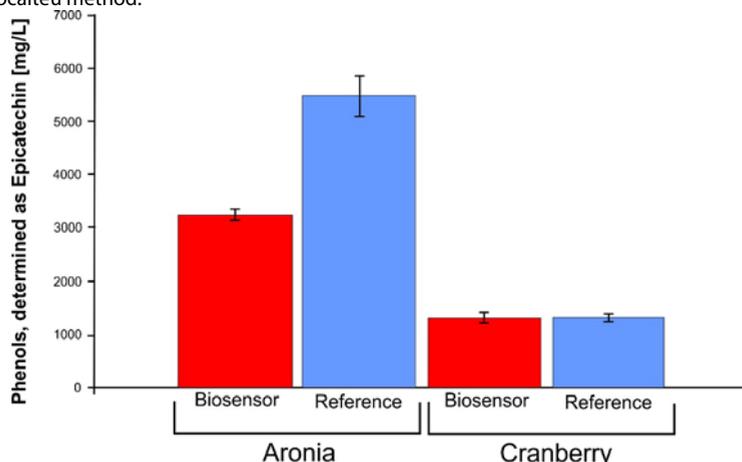
Fig. 5 Results for some commercially available juices. Reference values were obtained by the Folin-Ciocalteu method.

As a last example, cyanogenic glycosides can be determined by the enzyme cyanidase as shown in Fig. 2c (KETTERER, 2010). Again, ammonia is formed and the entire reaction causes a slight pH shift of the sample solution (KEUSGEN et al., 2004, TUREK et al., 2007, KETTERER AND KEUSGEN, 2010). Cyanogenic compounds are typical for the family *Rosaceae*, *Fabaceae* and the plants *Manihot esculenta* CRANTZ as well *Carica pap*aya L. On one hand, these compounds deliver an almond-like flavor, on the other hand, the liberated cyanide is highly toxic. The value of this biosensor is more

in the field of selecting species with a low amount of cyanogenic glycosides and decreased toxicity. The used recombinant cyanidase has been produced in *Escherichia coli* and is therefore available in reproducible quality and sufficient quantities (KEUSGEN et al., 2004).

In conclusion, it could be demonstrate that in some cases enzymatic biosensors can be considered as an alternative for chromatographic methods. Usually, not a distinct compound can be determined by theses sensors, but a class of secondary metabolites as shown by three examples. As a disadvantage, these sensors can be harmed by high concentrations of the analyte and improper storage conditions because of denaturation of the enzyme. But by experience it could be demonstrated that proper immobilization of the enzyme also increases stability of the enzyme. In any case, the sensor should be washed carefully with a suitable buffer after analysis and stored in a preserved buffer at +4 °C between measurements. This guarantees a lifetime of several months.

Acknowledgement

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