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Analysis of triterpenoids and phytosterols in vegetables by thin-layer chromatography coupled to tandem mass spectrometry

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ABSTRACT

Three TLC methods were used for an initial screening of some common plant triterpenoids and phytosterols in cuticular wax extracts of different vegetables (zucchini, eggplant, tomato, red pepper, mangold, spinach, lettuce, white-colored radicchio di Castelfranco, raddichio Leonardo, white cabbage, red cabbage and savoy cabbage). The preliminary experiments showed that the studied vegetables are potential sources of triterpenoids and phytosterols. To identify the compounds present in the extracts with high certainty, the first TLC–MS^2 method was developed for the analysis of eight triterpenoids (lupeol, α-amyrin, β-amyrin, cycloartenol, cycloartenol acetate, lupeol acetate, lupenone and friedelin) and two phytosterols (β-sitosterol and stigmasterol). This method takes the advantages of: (1) a satisfactory separation of the target compounds; (2) their differentiation according to the band colors; and (3) the potential of their discrimination by the acquired first-order mass (MS) and product ion (MS^2) spectra. Since the closely eluting compounds have complex and similar MS^2 spectra, distinguishing between them was possible by the proposed characteristic ions. Using a custom-built mass spectral library, the head to tail MS^2 spectra comparison of sample test solution zones and standard aided the compound identification. In addition to the molecular mass information, the developed atmospheric pressure chemical ionization method (APCI) in positive ion mode provided structural information, regarding the presence of functional group in the molecule. This approach resulted in many positively assigned compounds in the investigated vegetable waxes, from which more than a half are reported for the first time.

1. Introduction

Cuticular waxes constitute the waxy coverings of the plant organs and serve as plant protectants against unfavorable environmental conditions and insects. They are composed of long-chain aliphatic hydrocarbons, ketones, esters, fatty alcohols, fatty acids, aldehydes, as well as triterpenoids (C_{30}) and phytosterols (C_{18}–C_{30}) [1]. The last two groups of compounds present a very large and structurally diverse family of secondary plant metabolites, biosynthetically derived through the mevalonate pathway from six isoprene units (C_5H_8) [2]. They can exist as free compounds or in the form of esters and saponins. Pharmacological studies of triterpenoids showed anticancer, anti-inflammatory, anti-ulcerogenic, anti-microbial, anti-viral (including anti-HIV), anti-fungal, analgesic, antioxidative, hepatoprotective and some other activities [3–10]. Phytosterols are well known for their cholesterol-lowering properties, and also demonstrate anticancer, anti-inflammatory and immunoregulatory activities [11,12]. Due to the growing interest in triterpenoids and phytosterols, the development of suitable modern analytical methods for the determination of these metabolites in natural products, is of paramount importance.

Determination of triterpenoids in plant extracts is rather difficult, since many plants contain a vast amount of various triterpenoid compounds. Presence of isomeric triterpenoids in plant cuticular waxes renders the determination of triterpenoids even more difficult. Among the separation techniques, thin-layer (TLC) [13–16], supercritical fluid (SFC) [17], gas (GC) [18–24] and high-performance liquid (HPLC) [13,14,23–33] chromatography and capillary electrophoresis (CE) [34] have been used in their analysis. GC is favorable for the separation of positional triterpenoid isomers, and coupled to flame ionizaton (FID) [18,19,21–24] and mass spectrometric (MS) detection [18–20,22,24] has been widely used for their qualitative [18–20,22,24] and quantitative analysis [18,19,21–24]. A big disadvantage of this technique is the need of a prechromatographic derivatization, due to the non-volatility of the

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compounds, which additionally prolongs the analysis. On the other hand, HPLC reduces the sample pretreatment step by avoiding the derivatization. However, triterpenoids and phytosterols lack chromophores, which limits the mobile phase choice and reduces the sensitivity of UV detection [13,14,23,25]. Therefore, coupling HPLC with evaporative light scattering (ELSD) [24,26] or corona charged aerosol (CAD) detector [27] can be suitable, since they showed increased sensitivity compared to UV detectors for the compounds of interest. In addition, triterpenoids can be tagged with fluorescent groups and determined by fluorescence detector (FLD), as well [28,29]. However, UV–vis, as a non-selective and universal detector, is the most commonly used in HPLC. Furthermore, the mass spectrometry (MS) detector enables identification and quantification of compounds in real samples, and aids the structural elucidation of unknown compounds as well [13,14,26,30–33]. When product ion (MS^2) analysis is used in the identification of the triterpenoid positional isomers, the spectra differ only by the relative intensities of some mass peaks, which indicates that a good chromatographic separation prior to MS analysis is obligatory for unambiguous identification of the compounds [13,14]. Although, TLC offers lower separation efficiencies compared to GC and HPLC, it is a highly applicable technique especially for fast screening of compounds in various complex matrices simultaneously, since the sample purification step is usually avoided or is minimal. In addition, there are no limitations in the selection of mobile phase solvents in comparison to HPLC. Moreover, an increase in the specificity and sensitivity of the analysis can be achieved by its coupling to tandem MS [35].

To the best of our knowledge, there is a lack of methods for simultaneous identification or determination of common plant neutral triterpenoids and phytosterols. Moreover, there is scarce information on their presence in the cuticular waxes of various vegetables. Therefore, the main objective of the present study was to develop and apply a new TLC–MS^2 method for the analysis of the triterpenoids and phytosterols in vegetable extracts.

2. Experimental

2.1. Chemicals

All the solvents used in the study were at least of analytical grade. Dichloromethane, chloroform, *n*-propanol, *n*-hexane, ethyl acetate, acetic acid (glacial, 100%), sulfuric acid (95–97%), hydrochloric acid (fuming, 37%) sodium sulfate (anhydrous), potasium hydroxide and 4-methoxybenzaldehyde (anisaldehyde) were obtained from Merck (Darmstadt, Germany), while HPLC grade methanol and acetonitrile were produced by J.T. Baker (Deventer, The Netherlands). Acetone, LC–MS purity acetonitrile as well as reference standards for ursolic acid (≥90%) and β-sitosterol (≥97%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Other reference standards such as lupeol (≥99%), α-amyrin (≥98.5%), β-amyrin (≥98.5%), cycloartenol (≥90%), lupeol acetate (≥95%), cycloartenol acetate (≥90%), lupenone (≥95%), friedelin (≥99%) and betulinic acid (≥97%) were supplied by Extrasynthèse (Genay, France), while stigmasterol (≥99%) was obtained from Serva Feinbiohemica (Heidelberg, Germany) and oleanolic acid (≥97%) from Carl Roth (Karlsruhe, Germany). Ultrapure water was supplied by a Milli-Q water purification system (18 MΩ cm) from Millipore (Bedford, MA, USA).

2.2. Preparation of standard solutions

Stock solutions of all standards (1 mg mL^{-1}; except those of friedelin and betulinic acid with concentration of 0.1 mg mL^{-1}) were prepared in *n*-propanol and were further diluted with the same solvent to obtain working solutions (25 μg mL^{-1}). A mixture of all 13 standard solutions (MIX13, 25 μg mL^{-1}; lupeol, α-amyrin, β-amyrin, cycloartenol, cycloartenol acetate, lupeol acetate, lupenone, friedelin, ursolic acid, oleanolic acid, betulinic acid, β-sitosterol and stigmasterol) and a mixture with all the standards except ursolic, oleanolic and betulinic acids (MIX10, 25 μg mL^{-1}) were prepared by mixing 1 mL of each working solution, evaporating the solvent under nitrogen and redissolving the solid residue in 1 mL of *n*-propanol.

2.3. Preparation of vegetable extracts and sample test solutions

The extraction of the vegetable cuticular waxes and preparation of sample test solutions followed the procedure given in Ref. [13]. Fresh vegetables were purchased from a local market. Fruits from zucchini (*Cucurbita pepo* L., Cucurbitaceae; 1845 g), eggplant (*Solanum melongena* L., Solanaceae; 2775 g), tomato (*Solanum lycopersicum* L., Solanaceae; 4289 g) and red pepper (*Capsicum annuum* L., Solanaceae; 696 g) and leaves from mangold (*Beta vulgaris* L. ssp. *vulgaris* var. *cicla*, Chenopodiaceae; 77 g), spinach (*Spinacia oleracea* L., Chenopodiaceae; 194 g), lettuce (*Lactuca sativa* L. var. *capitata*, Cichoriaceae; 350 g), white-colored radicchio di Castelfranco (*Cichorium intybus* L. var *foliosum*, Cichoriaceae; 368 g), radicchio Leonardo (*Cichorium intybus* L. var. *foliosum*, Cichoriaceae; 529 g), white cabbage (*Brassica oleracea* L. subsp. *oleracea* convar. *capitata* L. var. *capitata* L. f. *alba*, Brassicaceae; 488 g), red cabbage (*Brassica oleracea* L. subsp. *oleracea* convar. *capitata* L. var. *capitata* L. f. *rubra*, Brassicaceae; 250 g), and savoy cabbage (*Brassica oleracea* L. subsp. *oleracea* convar. *capitata* (L.) Alef. var. *sabauda*, Brassicaceae; 157 g) were separately immersed into dichloromethane for 1 min. After addition of anhydrous sodium sulfate (1 g) to the extract (to bind the residual water), it was filtered through paper filter and the filtrate was concentrated under reduced pressure (Rotavapor, Büchi, Switzerland). The concentrated extract was transferred to a pre-weighted plastic tube (15 mL), and the solvent was evaporated to dryness by using a gentle stream of nitrogen. Dry wax residues (61–370 mg) were dissolved in chloroform to a concentration of 10 mg mL^{-1} and 1 mL of each vegetable extract was transferred in a separate autosampler vial. The extract solvent was evaporated to dryness and the solid residue was redissolved in *n*-propanol (1 mL) to give the final sample test solution. A hair dryer was used to speed up the solvation process of the waxy residues. The sample test solutions were cooled down to room temperature and filtered through a 0.45 μm Millipore Millex-HV hydrophilic poly(vinyldiene difluoride) (PVDF) membrane filter (Billerica, MA, USA).

2.4. Thin-layer chromatography

TLC was performed on the Merck 20 cm × 10 cm glass-backed HPTLC silica gel 60 (Art. No. 1.05641) and HPTLC C_{18} RP (Art. No. 1.05914) plates predeveloped with chloroform–methanol (1:1, v/v) and acetone, respectively, and dried in an oven at 110 °C for 30 min. Standard solutions, MIX10, MIX13 and sample test solutions were applied on the plates as 8 mm (or 6 mm for TLC–MS) bands, 10 mm from the bottom of the plates, by use of Linomat 5 (Camag, Muttenz, Switzerland). Plates used for compounds screening were developed to a distance of 8 cm (in 12 min) in a horizontal developing chamber (for 20 cm × 10 cm plates; Camag) using 6 mL of developing solvents *n*-hexane–ethyl acetate (5:1, v/v) for silica gel plates and acetone–acetonitrile (5:1, v/v) and ethyl acetate–acetonitrile (3:2, v/v) for C_{18} RP HPTLC plates [13]. For each case, 10 mL of the corresponding solvent was put in a tank for preconditioning (10 min).

As a part of the optimization of the separation for TLC–MS analysis, C_{18} RP HPTLC plates were developed by ethyl