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COMPARISON OF ULTRAVIOLET DETECTION AND CHARGED AEROSOL DETECTION METHODS FOR LIQUID-CHROMATOGRAPHIC DETERMINATION OF PROTOESCIGENIN

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Abstract: Escin, a complex mixture of pentacyclic triterpene saponins obtained from horse chestnut seeds extract (HCSE; *Aesculus hippocastanum L.*), constitutes a traditional herbal active substance of preparations (drugs) used for a treatment of chronic venous insufficiency and capillary blood vessel leakage. A new approach to exploitation of pharmacological potential of this saponin complex has been recently proposed, in which the β-escin mixture is perceived as a source of a hitherto unavailable raw material, pentacyclic triterpene aglycone – protoescigenin. Although many liquid chromatography methods are described in the literature for saponins determination, analysis of protoescigenin is barely mentioned. In this work, a new ultra-high performance liquid chromatography (UHPLC) method developed for protoescigenin quantification has been described. CAD (charged aerosol detection), as a relatively new detection method based on aerosol charging, has been applied in this method as an alternative to ultraviolet (UV) detection. The influence of individual parameters on CAD response and sensitivity was studied. The detection was performed using CAD and UV (200 nm) simultaneously and the results were compared with reference to linearity, accuracy, precision and limit of detection.

Keywords: protoescigenin, UHPLC, charged aerosol detection

Abbreviations: CAD – charged aerosol detection, HCSE – horse chestnut seeds extract, UHPLC – ultra-high performance liquid chromatography

Analysis of saponins is very challenging task because of high degree of structural complexity, multiplicity of similar functional groups and resulting physicochemical likeness of individual compounds, as well as the lack of chromophores, which hampers sensitivity of popular UV based detection systems. Although all analytical studies involving saponins – phytochemical, environmental, clinical and food research use contemporary separation techniques, the state of art in particular disciplines differs considerably: e.g., phytochemical research is mainly concerned with structural analysis of new compounds, while quantification and specification of plant materials seem to be a secondary issue. Therefore, even incremental improvements in qualitative and quantitative analysis of saponins come handy for pharmaceutical and clinical evaluation, as clearly reflected by regularly reported recent advancement (1, 2).

Escin (or aescin), a complex mixture of acylated pentacyclic triterpene saponins obtained from HCSE, is used as a phlebotropic drug for a treatment of chronic venous insufficiency and capillary blood vessel leakage. Recently, a new approach to exploitation of the escin mixture has been proposed (3). In this approach the saponin mixture was subjected to consecutive chemical transformations designed and developed as a scalable, validated technical processes, to afford hitherto unavailable material protoescigenin (**1**, **PES-01** see Scheme 1) in state of high chemical purity. Protoescigenin appears to be a suitable starting material for the design and further synthesis of various saponin mimetics (4). It was selected as the first candidate molecule, because although known as chemical entity from the classical period of triterpene saponin exploration (5, 6) and more recently confirmed as 3β,16α,21β,22α,24,28-hydroxyolean-12-ene by modern analytical and spectral tools (7), it is not commercially available and its chemistry is practically unexplored.

Analysis of the starting materials quality constitute a testing practice, that is essential for manu-

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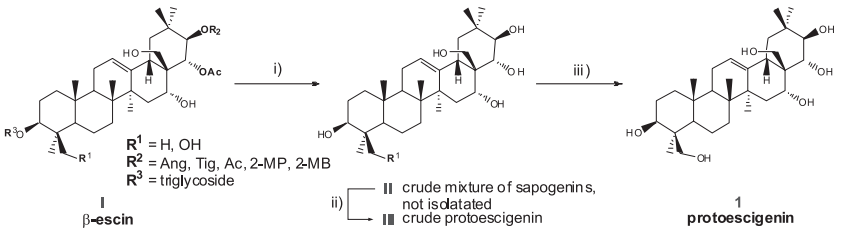

Scheme 1. Original method of preparation of protoescigenin (**1, PES-01**) from â-escin (**I**). Conditions: i) a) H_2SO_4, MeOH, reflux, b) KOH, H_2O; ii) MTBE, MeOH, H_2O; iii) a) *i*-PrOH, H_2O, b) *i*-PrOH, cyclohexane; Ang- angeloyl, Tig – tigloyl, Ac – acetyl, 2-MP – 2-methylpropanoyl, 2-MB – 2-methylbutanoyl (3)

facturing process development and helps to ensure product quality and consistency. Establishing of starting material specification, defined as a set of tests, references to analytical methods and appropriate acceptance criteria, is also the part of this practice. Among analytical methods, high performance liquid chromatography (HPLC) with UV detection has been commonly used as the technique for purity and assay control. However, traditional HPLC methods are frequently time- and solvent-consuming, especially in case of analysis of plant extracts samples (8). Additionally, for compounds lacking strong UV chromophores (like saponins) the most popular and widely used ultraviolet detection can be elusive. In such situations ultrahigh-performance liquid chromatography (UHPLC), accepted as a key approach to getting more analytic information in less time, with solvent reduction and waste minimization, can be a technique of choice. The solution to the detection problem may be the use of alternative detection method, like the charged aerosol detection, CAD. The response generated by CAD is independent of the chemical and spectral properties of non-volatile analytes but at the same time is mass-dependent, offering similar responses for the same mass of analyzed compounds (9–11). CAD system has many advantages, like broad dynamic response range up to 4 orders of magnitude, good precision, availability in a versions designed for both, classical HPLC and fast, UHPLC, as well as simple and reliable operation (9). However, CAD – as detector employing a nebulization process – has also some limitations: the response depending on the composition of the mobile phase and lack of possibility of certain peak identification or peak purity analysis (no spectral information is acquired).

Although many methods have been described in the literature for separation and analysis of saponins, HPLC coupled with various detectors being the most commonly used (12–15), none of them relates directly to the determination of protoescigenin. Additionally, applications of charged aerosol detection method for analysis of this group of compounds are still very rare. Until now, only several works and papers covering this subject have been published (12, 14, 16–19). The aim of our work was to fill the gap in this research area, and as a result a fast and universal method for quantitative analysis of protoescigenin was developed. A new UHPLC method with dual detection system, UV (200 nm) and CAD, was applied. The detection was performed using both systems simultaneously and the results were compared with reference to linearity, accuracy, precision and limit of detection and quantification. To the best of our knowledge, this work is the first in which dual detection system (UV/CAD) has been applied for protoescigenin determination.

EXPERIMENTAL

Chemicals and reagents

Samples of protoescigenin, including working standard (99.3%, UHPLC), as well as its main impurity barringtogenol C (BAC, 86.6% purity according to UHPLC) were manufactured and tested in Pharmaceutical Research Institute (Warszawa, Poland). Reference standards were fully characterized using the following techniques: UHPLC, ^{1}H NMR, ^{13}C NMR, IR, XRPD, MS, TG and DSC.

Acetonitrile (ACN) and methanol (MeOH) of HPLC grade were purchased from POCH S.A. (AVANTOR Gliwice, Poland). Acetic acid (HAc, ≥ 99.8%) was obtained from Fluka (Sigma-Aldrich, USA). Demineralized water (≥ 18.0 W/cm) from Barnstead system (Thermo Scientific, USA) was produced in the laboratory.

Chromatography

An UltiMate 3000RS UHPLC system (Thermo Scientific, Sunnyvale, CA, USA) was used to perform all of the analyses. The instrument was equipped with autosampler (WPS-3000TRS) and

column oven (TCC-3000RS), both enabled cooling. DAD-3000RS photodiode array and Corona charged aerosol (CAD) detectors (Thermo Scientific, Sunnyvale, CA, USA) were connected in this system. The data were analyzed using the Chromeleon software package.

The chromatographic separations were performed on an Acquity C18 BEH column (50 × 2.1 mm i.d., particle size 1.7 μm) manufactured by Waters (Waters Corporation, Milford Massachusetts, USA). The column oven temperature was set at 35°C and autosampler was kept at 20°C. The binary mobile phase, which composed of acetonitrile and 0.1% acetic acid (30 : 70, v/v), was pumped at flow rate of 0.4 mL/min. UV (DAD) detection was performed at 200 nm and the injection volume was set as 1 μL. Examined samples were dissolved in methanol (c ~ 2 mg/mL). CAD detection was performed with nitrogen pressure of 35 psi and range of 100 pA. The UV and CAD chromatograms were scanned concurrently.

Preparation of sample solutions

The stock solutions of standard and samples were prepared by dissolving 20.0 mg of substance into 10 mL of methanol. Stock solutions were further diluted by methanol to achieve a proper concentration for measurements and to get individual points of calibration curves for two ranges: (1) broad, from 0.002 mg/mL to 2.40 mg/mL and (2) narrow, for five concentration levels (i.e., from 1.60 to 2.40 mg/mL).

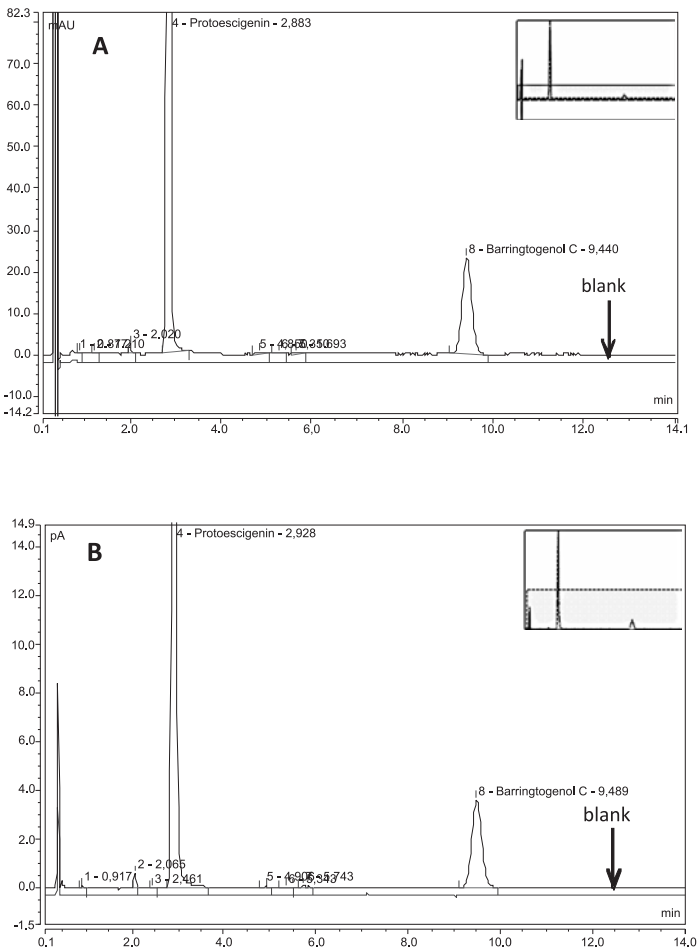

Figure 1 Example chromatogram of protoescigenin sample and blank: **A**. UV detection (200 nm), **B**. CAD detection

RESULTS AND DISCUSSION

Optimization of chromatographic conditions – UV detection and charged aerosol detection

The separation of protoescigenin and its process-related impurities was achieved with 30% of acetonitrile (ACN) in the mobile phase. This ACN concentration was chosen as an optimal for a good resolution of all observed peaks within reasonable time period (see Fig. 1). Because volatile additives are recommended for CAD detection, a couple of various eluents were tested as the aqueous part of the mobile phase during method development. Due to the poor UV absorbance of triterpene glycosides, 200 nm as the optimal wavelength in UV (DAD) detection of protoescigenin was selected.

Mobile phase composition

Various additives in the aqueous part of the mobile phase and their influence on the main peak area was examined. Ammonium acetate buffer, trifluoroacetic acid, formic acid and acetic acid, all at different concentrations, were tested. The results are presented in Figure 2 (both, for UV and CAD).

In case of UV, detection 10 mM concentration of ammonium acetate resulted in the lowest peak areas, changing concentration of this buffer had also noticeable influence on the UV response. Using of 5 mM ammonium acetate gave almost similar results as in the case of 0.1% trifluoroacetic acid (TFA). For both, TFA and ammonium acetate, lower concentrations resulted in the higher peak areas. Changing the concentration of formic acid and

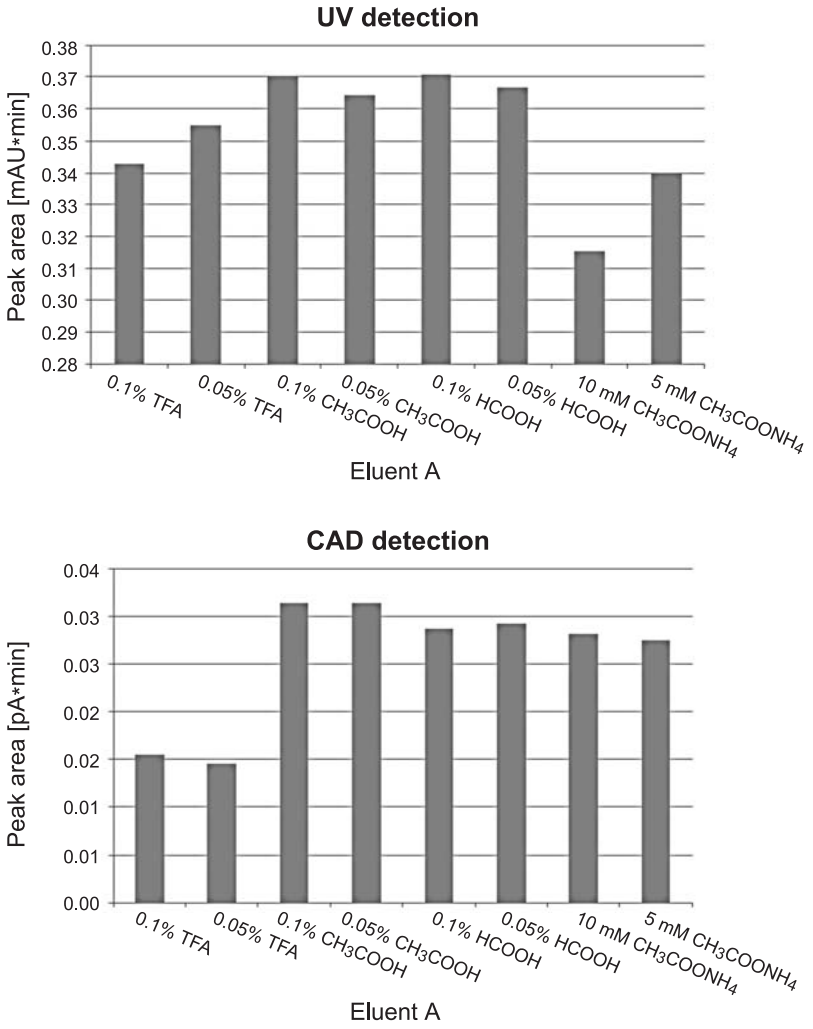

Figure 2 The influence of various additives to UV and CAD response (concentration of tested sample ~1 mg/mL, inj. vol. 1 µL)

acetic acid did not affect the UV detector response; in case of these two additives the highest peak areas of protoescigenin were observed.

Different results were obtained in case of CAD detection. TFA, in both concentrations (0.1 and 0.05%), had the strongest influence on the response (the lowest peak areas were observed); additionally a very strong increase of spike peaks was observed. On the other hand, using of ammonium acetate, as well as acetic and formic acid gave comparable CAD response, independent of the additive concentration, with acetic acid giving the highest peak areas.

Based on obtained results, as optimal component of the water part of the mobile phase, for both detection systems, 0.1% acetic acid was selected.

Influence of the flow rate on UV and CAD response

During the method development it was observed that for UV detection the protoescigenin peak area decreases with increasing mobile phase flow-rate, whereas CAD response remains flow rate-independent. This finding has been confirmed on two examples: (1) for 0.1% acetic acid/acetonitrile (70 : 30, v/v) and (2) 0.1% formic acid/acetonitrile (70 : 30 v/v) as a mobile phase. Example (1) is illustrated in Figure 3.

The explanations for the reduction of the signal intensity are described in the literature (20, 21). Because UV detector is concentration-sensitive, its signal follows the Lambert-Beer law (absorbance = concentration × molar abs. × optical path length).

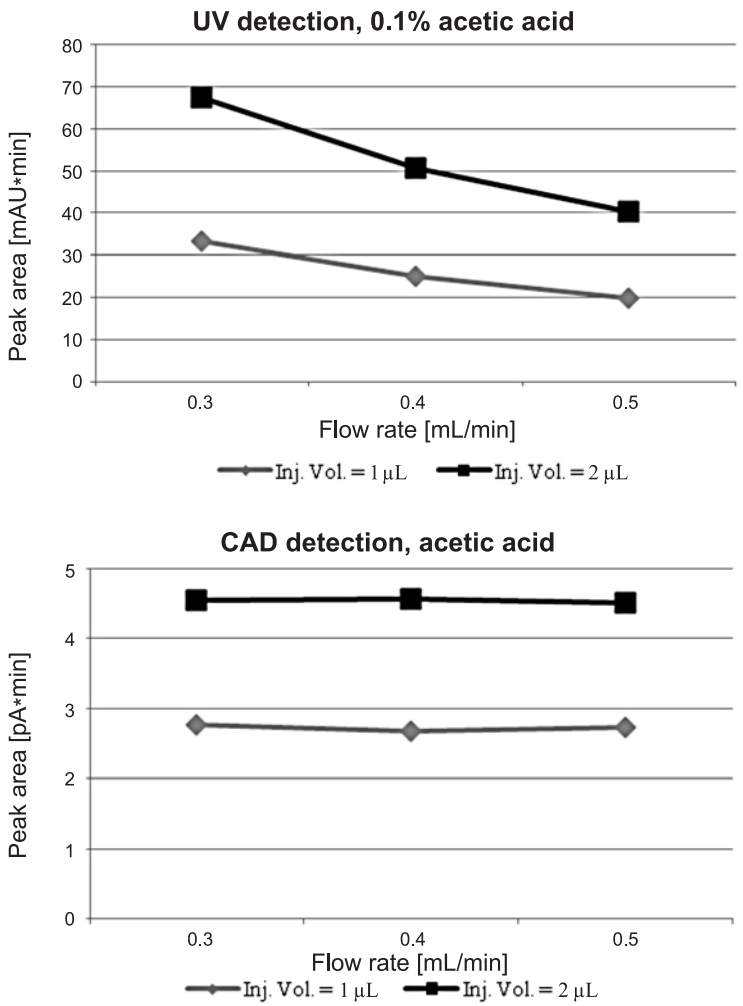

Figure 3 Correlation between the detector response and the mobile phase flow rates

When a peak reaches the cell of an UV detector its width is equal to its residence time, whereas its height is governed by the analyte concentration at the peak minimum. As a consequence, the peak area depends very strongly on the flow rate of the mobile phase (20, 21). On the other hand, charged aerosol detector as mass-sensitive is unaffected by this phenomena.

Influence of sample concentration and injection volume on S/N value in UV and CAD

Optimal sample concentration and injection volume was determined in both detection approaches. For protoescigenin concentration of 1 mg/mL and injection volume of 2 µL, S/N ratios for CAD were two times higher than for the UV detection.

Results are presented in Figure 4.

System suitability test (SST) and validation

The SST was performed by 7 subsequent injections of protoescigenin (PES-01) sample containing several process-related impurities, among them barringtogenol C (BAC) at the level of ~17% (normalization calculation method). Parameters such as peak asymmetry, resolution between critical peaks and repeatability (based on retention times and peak areas, the repeatability expressed as RSD %) were established. SST results were compared for both detection approach (see Table 1). Results are similar for both detection systems, but in case of CAD better symmetry of PES-01 peak was achieved.

A typical chromatogram is shown in Figure 1.

Limit of detection (LOD) and limit of quantitation (LOQ)

In analytical methods the detection limit (LOD) is defined as the minimum level at which the analyte can be reliably detected and quantitation limit (LOQ) as the minimum level at which the analyte can be quantified with acceptable accuracy and precision (22). Several approaches for determining the LOD and LOQ are possible – in this work, method based on signal-to-noise ratio (S/N) was used. Limits of detection and quantitation for both detection systems were established, assuming that S/N ratio for LOD should be not less than 3.3 and for LOQ not less than 10. The LOD criterion was fulfilled for the solutions at the concentration of 0.001 mg/mL for both, UV and CAD detection. In the case of CAD, S/N values were found to be almost two times higher than for the UV detection. The same was observed for LOQ, where the criterion was fulfilled for the solutions of protoescigenin with concentration of 0.002 mg/mL.

Linearity and calibration range

The linearity of methods (UV and CAD) was evaluated in two ranges of concentration: (1) broad, from LOQ to 120% of protoescigenin (i.e., from 0.002 mm/mL to 2.40 mg/mL) and (2) narrow, for five concentration levels (80% – 120%, i.e., from 1.60 to 2.40 mg/mL). Results are presented in Table 1. The obtained calibration curves were linear in both defined ranges. However, in the case of CAD, a log-log transformation for calibration curve, which

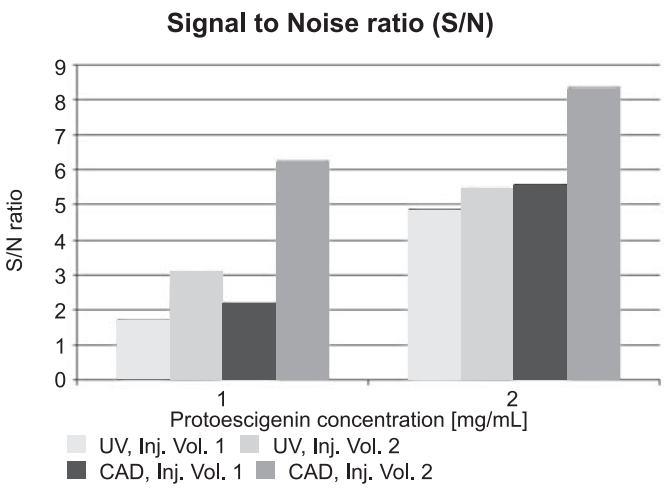

Figure 4. Influence of sample concentration and injection volume on S/N value in UV and CAD

Table 1. Method validation results.

	UV (200 nm)	CAD	Limits for pharmaceutical substances
Accuracy [%]	99.95	98.97	Recovery 98–101%
Precision [%RSD]	0.48	0.37	RSD ≤ 1.0%
Linearity – from LOQ to 120% (0.002 – 2.408 mg/mL)	R^2 = 0.9995 y = 0.4128x + 0.4000	R^2 = 0.9990 y = –0.044x + 1.042x + 0.713	$R^2 \geq 0.998$
Linearity – from 80–120% (1.616 – 2.408 mg/mL)	R^2 = 0.9996 y = 0.4297x – 1.4218	R^2 = 0.9986 y = 0.0347x + 0.5925	$R^2 \geq 0.998$
LOD S/N	0.001 mg/mL 4	0.001 mg/mL 7	S/N ≥ 3
LOQ S/N	0.002 mg/mL 11	0.002 mg/mL 21	S/N ≥ 10
System suitability results:			
Asymmetry	0.81	0.96	$0.8 \leq A_s \leq 1.5$
Resolution between PES-01 and the closest impurity	4.30	3.42	$R_s \geq 1.50$
PES-01 Peak area, RSD %	0.62	0.74	RSD ≤ 1.0%
PES-01 Retention time, RSD %	0.13	0.12	RSD ≤ 1.0%
BAC Retention time, RSD %	0.11	0.11	–

judgment concurred with those of other reports (23, 24), was necessary – but only for the broad concentration range. It was concluded that the developed method is appropriate for quantitative purposes for both UV and CAD detection, in broad concentration range.

Accuracy and precision

Method precision was tested using six protoescigenin samples at 100% of concentration (c = 2 mg/mL) and was expressed as % RSD. Accuracy was determined on three levels: 80%, 100% and 120%, three sample solutions per each level, and was expressed as % of recovery (see Table 1). All the results were in correspondence with the requirements for method validation.

CONCLUSIONS

Protoescigenin, the main genin of HCSE, was selected as a substrate for further exploratory chemistry. Physicochemical characterization of this compound has been recently described by Gruza et al. (3). As a complement to the aforementioned work, a new, fast and universal, ultra-high performance liquid chromatography (UHPLC) method for quantitative analysis of protoescigenin has been developed and validated. In the method dual detection system has been applied, with using UV (200 nm) and CAD simultaneously.

The results achieved in method optimization showed an influence of the mobile phase flow-rate on UV detector response, expressed as decrease of the protoescigenin peak area with increasing flow rate value. A relationship between the type of the additive to the aqueous mobile phase and both UV and CAD detectors response was also noticed. The best results were obtained when 0.1% acetic acid was used as the additive.

The SST and validation results were in good agreement with validation requirements for both detectors. UV and CAD detectors gave linear response, both for the narrow and the broad range of concentrations. The sensitivity of CAD detection was a little bit greater than the UV, when comparing achieved S/N values.

Combining of the two detection systems – classical UV (in version of DAD detector) and CAD is the big advantage of the developed method, because both detection approaches complement each other. CAD allows for slightly better detection of weakly UV active protoescigenin and on the other hand, the lack of possibility of peak purity analysis in case of CAD is solved by simultaneously employ-

ing of DAD detector. The results obtained for both detection approaches and their usefulness for protoescigenin quantification have been demonstrated.

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