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Journal of Chromatography B, 770 (2002) 283–289

JOURNAL OF CHROMATOGRAPHY B

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Liquid chromatographic analysis of supercritical carbon dioxide extracts of *Schizandra chinensis*

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Abstract

Six major lignans (schizandrin, gomisin A, deoxyschizandrin, γ-schizandrin, gomisin N, wuweizisu C) in the caulomas and leaves of *Schizandra chinensis* (Turcz.) Baill., and cinnamic acid in the leaves of the plant, were quantitatively analysed by high-performance liquid chromatography in reversed-phase mode with UV detection. Resolution of the determined lignans was evaluated for two multistep gradients applied. Samples for HPLC analysis were prepared by extraction with supercritical carbon dioxide at pressures of 20–27 MPa and temperatures of 40–60 °C. Kinetics of the extraction of individual components was measured and simulated with a model.

Keywords: *Schizandra chinensis*; Carbon dioxide

1. Introduction

Fruits and seeds of *Schizandra chinensis* (Turcz.) Baill. are used in Oriental medicine as stimulants and adaptogenic drugs. They also have antioxidative and anti-inflammatory properties. Furthermore, they affect the cardiovascular system, the stomach and intestinal tract. Their hepatoprotective effect is very important, too. Biologically active components of the drug are lignans, compounds with a dibenzo-[*a*,*c*]cyclooctadiene skeleton [1]. Lignans are also present in other parts of the plant.

The lignans in *S. chinensis* have been most frequently analysed using high-performance liquid chromatography in reversed-phase mode (RP-HPLC) with UV detection [2–9]. The content of major lignans schizandrin, gomisin A, deoxyschizandrin, and gomisin N in the drug, and also of lignan wuweizisu C, was determined by RP-HPLC using an isocratic elution and mobile phases methanol–water [2,3], or acetonitrile–water [4,5]. Other combinations of lignans in the drug were also analysed using isocratic elution with methanol–water [6] and with acetonitrile–water–acetic acid [7]. Gradient methods have been applied to increase resolution of lignans. The content of a total of 10–11 lignans was determined using a one-stepwise gradient elution with mobile phase acetonitrile–methanol–water [8,9].

A total of 15 lignans contained in the fruits were separated and identified by electrospray high-performance liquid chromatography–mass spectrometry coupled with photodiode-array detector. The separation in reversed-phase mode was performed using gradient elution with methanol–water [10]. Recently, capillary electrochromatography using polymer-based monolithic stationary phase has been de-

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1570-0232/02/$ – see front matter
PII: S1570-0232(01)00634-1

veloped and successfully applied to analyse and quantify the lignans from seeds [11].

Supercritical carbon dioxide (SC-CO_2) as a more environmentally friendly alternative to organic solvents has been applied to extract lignans from seeds [2,3,11], fruits [3–5,7], stems (caulomas) [5], and leaves [2,5] of *S. chinensis*. With a sufficient time of extraction the recovery was almost complete except for the leaves, where only 26–37% of lignans was extracted with pure CO_2 due to a strong interaction of lignans with plant matrix. The recovery increased to 87% when carbon dioxide was modified by addition of 10% of ethanol [5]. The kinetics of CO_2 extraction of individual lignans from the plant has been studied in detail only for deoxyschizandrin from the fruit [7].

In this study, the extraction with SC-CO_2 was applied to obtain lignans from caulomas and leaves of *S. chinensis*. Six lignans, namely schizandrin $C_{24}H_{32}O_7$ (I), gomisin A $C_{23}H_{28}O_7$ (II), deoxyschizandrin $C_{24}H_{32}O_6$ (III), γ-schizandrin $C_{23}H_{28}O_6$ (IV), gomisin N $C_{23}H_{28}O_6$ (V) and wuweizisu C $C_{22}H_{24}O_6$ (VI) were determined in the extracts. Their structures established by means of spectral ^{13}C NMR studies [1] are available (Fig. 1). HPLC separation in reversed-phase mode was applied to determine extraction yields of individual lignans dependent on extraction time. Moreover, co-extraction of cinnamic acid from leaves was measured. Kinetics of extraction of individual components was measured and fitted to a model. The SC-CO_2 extraction yields were compared with those obtained from the drug by extraction with boiling ethanol.

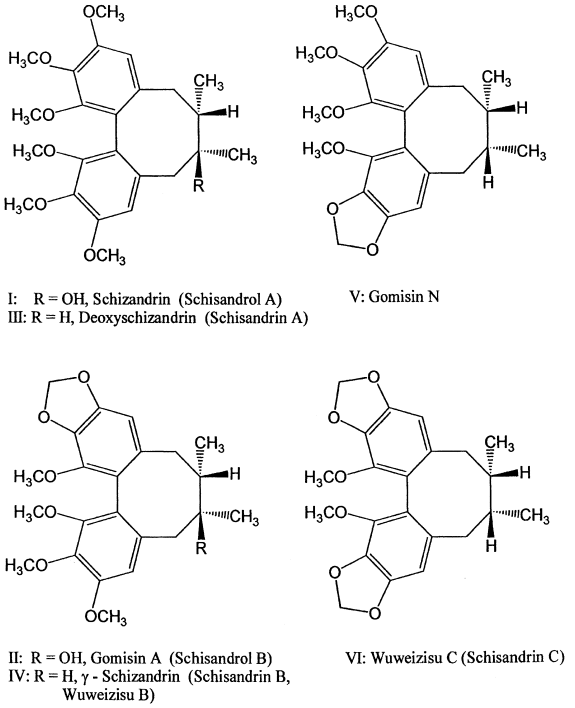

Fig. 1. Structural formulas of the lignans I–VI. Alternative names of lignans are given in parentheses.

2. Plant material

Caulomas and leaves of *S. chinensis* were collected in July 2000 from a 13-year-old population founded in northern Bohemia with seeds originating from Hortus Botanicus Centri Scientiarum Extremiorientalis Academiae Scientiarum, Vladivostok, Russia. A voucher specimen (Herbarium No. 00-26-03-01) was deposited in the herbarium of the Department of Pharmaceutical Botany and Ecology, Faculty of Pharmacy, Hradec Králové. They were dried at room temperature and ground to particles smaller than 0.4 mm.

3. Chemicals

Acetonitrile for HPLC, 99.93% and acetonitrile for UV-spectroscopy (both Fluka, Buchs, Switzerland), water for HPLC, methanol for UV spectroscopy, prepared from methanol p.a. (Lachema, Neratovice, Czech Republic) by rectification on the column, and carbon dioxide, 99.7% (Linde Technoplyn) were used as solvents. Pure lignans I–VI isolated at the Faculty of Pharmacy served as standards.

4. Experimental procedures

4.1. HPLC analysis

Two chromatographic sets and columns were used for the analysis.

4.1.1. Apparatus 1

The HPLC set consisted of the HP 1090, the ultraviolet diode array detector and refractometer (Hewlett-Packard, Waldbron, Germany). The analytical column EC 250×4 mm I.D., the stationary phase Nucleosil 100 5 μm C_{18} endcapped (Macherey-Nagel, Düren, Germany) and the guard column 8×4 mm I.D., Nucleosil 100 5 μm C_{18} (Macherey-Nagel, Düren, Germany) were used. Dead volume of the column was determined using D_2O and NaCl.

Chromatographic conditions were as follows: the ambient temperature was 18–20 °C, the flow rate of the mobile phase was 0.75 ml min^{-1}, and UV detection was performed at λ=254 nm.

Multistep gradients were used:

Gradient A. CH_3CN–H_2O: 5 min 50:50 (v/v) isocratic; gradient 30 min 60:40 (v/v); gradient 20 min 70:30 (v/v); 15 min 70:30 (v/v) isocratic.

Gradient B. MeOH–H_2O: 1 min 70:30 (v/v) isocratic; gradient 34 min 95:5 (v/v); 5 min 95:5 (v/v) isocratic.

The samples were dissolved in 2 ml of acetonitrile, filtered through a cartridge filled with silica gel and stationary phase C_{18} (Tessek, Prague, CR), and further diluted, if necessary.

The external standard method was applied for the quality and the quantity HPLC analyses of the SC-CO_2 extracts of lignans.

4.1.2. Apparatus 2

The apparatus consisted of a PV 4100 Liquid Chromatograph (Philips), PV 4110 UV–Vis Detector (Philips) and Pye Unicam PV 4021 multichannel detector (Philips). The column MERCK-Lichrospher 100 RP 18, 5 μm, 250×4 mm I.D. (Merck, Darmstadt, Germany) and the pre-column Tessek–CGC SGX C_{18}, 10 μm, 30×3 mm I.D. were applied.

Chromatographic conditions were as follows: temperature of 25 °C, flow rate 0.5 ml min^{-1}, injection volume 20 μl, UV detection at λ=254 nm, mobile phase acetonitrile–water 40:60 (v/v) to 70:30 (v/v), gradient 60 min, consecutively 70:30 (v/v), 30 min, isocratic elution.

The external standard method was applied for the quality and the quantity HPLC analyses.

The extracts were dissolved in the solvents (for ethanolic extracts, mixture acetonitrile–water 85:15 (v/v) was used, extracts from supercritical extraction were dissolved in methanol, 50 mg dry matter/1 ml solution), sonicated for 30 min (Sonorex Super 10P, frequency 35 kHz, grade 5), the resulting solution was passed through the SPE column and eluted by the solvent up to a final volume of 5.00 ml.

4.2. Supercritical fluid extraction

The SC-CO_2 extracts were obtained under pressure ranging from 20 to 27 MPa and temperature from 40 to 60 °C. A known quantity of caulomas or leaves (4–5 g) was extracted with SC-CO_2 (extractor 12 ml, 8 mm I.D.; 20 and 27 MPa; 40, 50, and 60 °C; 0.25–0.5 l min^{-1} CO_2, measured at ambient conditions). Dynamic extraction followed after 10–15 min of static extraction; the solvent flowed from the top to the bottom of the extractor. The extract was collected in periodically exchanged traps behind a heated micrometer valve where the solution was expanded to atmospheric pressure.

4.3. Liquid solvent extraction

The mixture of dry vegetative parts (about 0.5 g accurately weighed) and 95% ethanol (50 ml) was refluxed for 30 min, filtered, and the solvent removed under reduced pressure. The residue was then dried under vacuum for 24 h (exsiccator, phosphorus pentoxide).

5. Results and discussion

5.1. HPLC analysis

The chromatogram of SC-CO_2 extract is shown in Fig. 2. The resolution of the determined components was calculated according to the equation [12]

$$R_{i,j} = \frac{\sqrt{n}}{4}\left(\frac{r_{j,i}-1}{r_{j,i}+1}\right)\left(\frac{k}{k+1}\right)$$

where the capacity factor, *k*, and the number of theoretical plates of the column, *n*, are arithmetical means of the values calculated separately for the component (peak) *i* next to the component *j* in the chromatogram, and $r_{j,i}=k_j/k_i$ is the retention ratio. The resolution values are listed in Table 1.

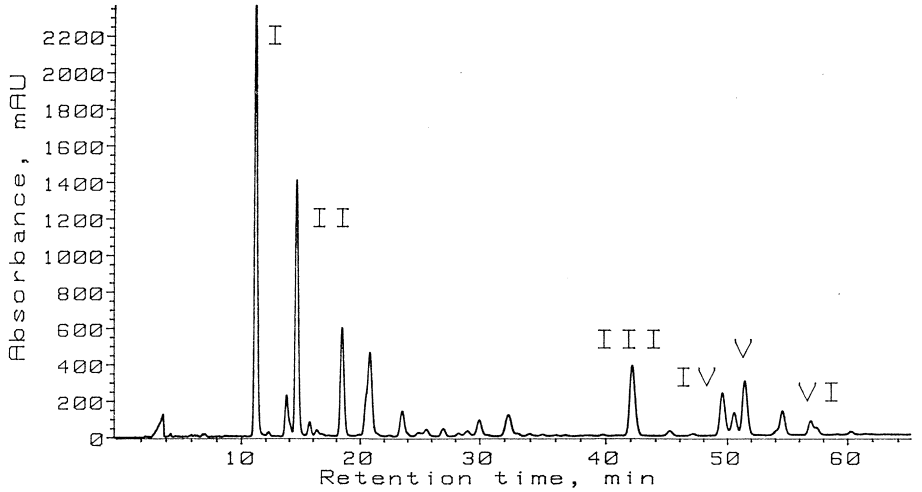

Fig. 2. Chromatogram of the SC-CO_2 extract from caulomas obtained in the initial extraction period. HPLC column EC 250×4 mm, Nucleosil 100-5 C_{18}. Guard column 8×4 mm, Nucleosil 100-5 C_{18}. Mobile phase acetonitrile–water. Gradient A, flow rate 0.75 ml min^{-1}, UV detection at $\lambda = 254$ nm.

Although acetonitrile in Gradient A was a very good mobile phase modifier, splitting of the peaks was observed occasionally. The splitting was most probably caused by dissolving the sample in a solvent of higher elution power than is the power of the mixture of solvents efficient when the separation begins. The difference in solvent power results in a partial and temporary precipitation of the substance in the column, which manifests by the splitting. Using Gradient B, the resolution of the lignans IV and V was slightly worse, but no peak splitting occurred.

The column in Apparatus 2 was calibrated for all six lignans. The calibration graphs were linear over the studied concentration range. The column in Apparatus 1 was calibrated for lignans IV and VI, and for the remaining lignans (I–III and V) the ratio of their calibration line slope to the calibration line slope of lignans IV and VI, as determined on the column in Apparatus 2, was applied.

5.2. SC-CO_2 extracts

The extraction process consisted of an initial period of rapid extraction followed by a second period of slow extraction (Fig. 3). Under the experimental conditions, the analyte concentration in the effluent in the first extraction period was independent of the flow rate. This indicates that the eluate was saturated with analytes.

Table 1
Resolution ($R_{i,j}$) of the lignans (I–VI), the compound (peak) *i* next to the compound *j* in the chromatogram, and number of theoretical plates of the column (*n*)

Compounds separated *i*, *j*	$R_{i,j}$		*n*	
	Gradient A	Gradient B	Gradient A	Gradient B
I, II	3.86	6.90	13 436	23 626
II, III	20.29	16.66	28 153	46 633
III, IV	4.45	–	50 026	–
III, (IV + V)	–	0.93	–	13 437
IV, V	1.22	–	63 782	–
(IV + V), VI	–	3.78	–	174 362
V, VI	3.19	–	64 218	–

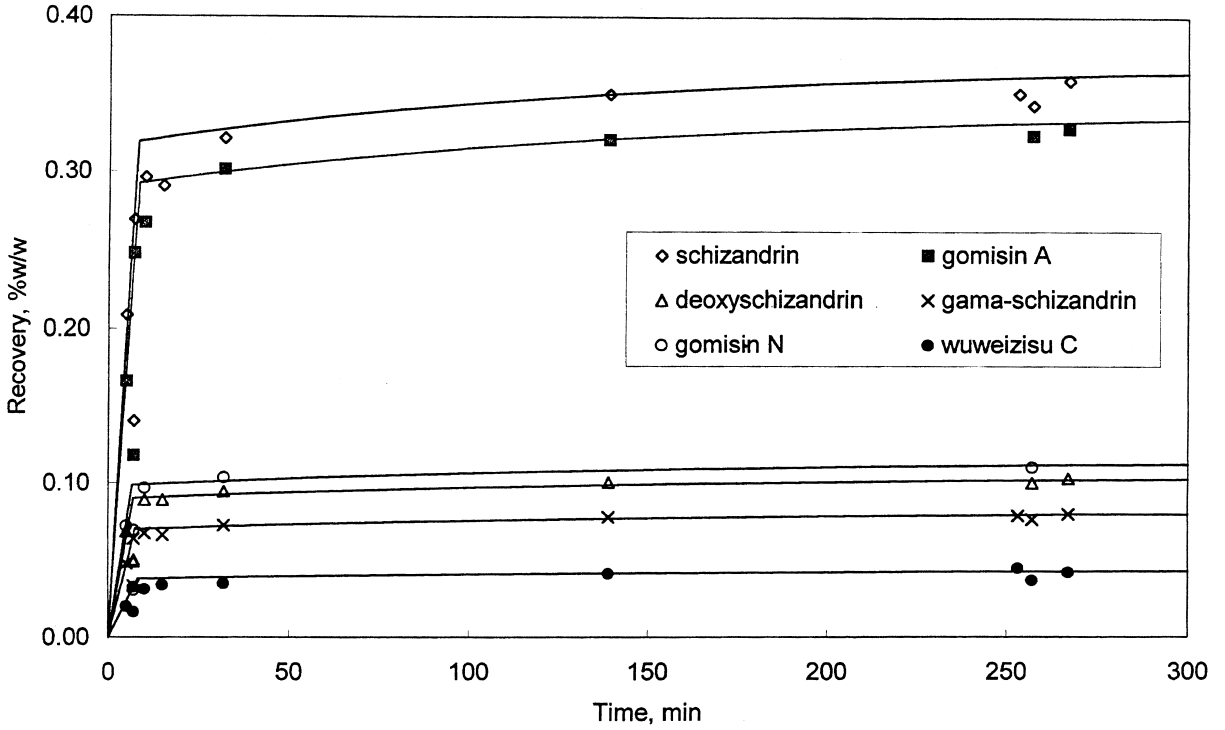

Fig. 3. Extraction curves of lignans from caulomas: results of three experimental runs with identical conditions (27 MPa, 50 °C, 0.9 g min^{-1} CO_2). Full lines were calculated using the model equations.

5.2.1. Extraction from caulomas

Experimental extraction curves were fitted to a model distinguishing between the fraction z of the plant tissue that became easily accessible for the extraction as a result of grinding and the fraction $1-z$ of the tissue that remained intact. The extraction from the intact tissue is controlled by inner diffusion. In the first period, the solvent is saturated with analyte:

$$e_i = k_{0,i}qt \text{ for } e_i \le e_{z,i} = ze_{\infty,i},\ t \le t_{z,i} = \frac{ze_{\infty,i}}{k_{0,i}q}$$

where e_i is the extraction yield of the ith component (related to dry mass of caulomas), $k_{0,i}$ is the mass ratio of the ith component to CO_2 in the saturated solution, q is the solvent specific flow rate (g CO_2/g caulomas, dry mass), t is the extraction time, $e_{\infty,i}$ is the maximum extraction yield, and $e_{z,i}$, $t_{z,i}$ are the values of yield and time at the end of the first extraction period. The rest of the analyte is extracted in the second period:

$$e_i = ze_{\infty,i}\{1-(1-z)\exp[-k_1(t-t_{z,i})]\}$$
$$\text{for } e_i > e_{z,i},\ t > t_{z,i}$$

where k_1 is the rate constant for the second extraction period. Model parameters were $z = 0.86$, $k_1 =$ 0.002 min^{-1} at 40 °C, 0.007 min^{-1} at 50 °C, and 0.008 min^{-1} at 60 °C, the maximum yields $e_{\infty,i}$ are listed in the first line of Table 2. The saturated concentrations in the initial extraction period, $k_{0,i}$, were both temperature- and pressure-dependent. Their highest values were achieved at 50 °C and 27 MPa, when the total mass ratio of major lignans to the solvent was $k_0 = \Sigma k_{0,i} = 0.12\%$ w/w.

5.2.2. Extraction from leaves

The extraction curves measured for lignans and cinnamic acid were approximated by two straight lines (Fig. 4). As the extraction yield at their crossing point strongly increased with extraction temperature, the process in the second extraction period was controlled rather by the analyte–matrix

Table 2
Extraction yields (% w/w dry mass) of lignans from caulomas and leaves of *S. chinensis* (Turcz.) Baill. Comparison of the solvent efficiencies

Dry material	Solvent	Lignan					
		I	II	III	IV	V	VI
Caulomas	SC-CO_2	0.37	0.34	0.11	0.082	0.12	0.045
	Ethanol	0.097	0.19	0.036	0.040	0.093	0.047
Leaves	SC-CO_2	0.072	0.049	0.032	0.017	0.032	0.012
	Ethanol	0.041	0.057	0.033	0.029	0.057	0.025

interaction than by the diffusion of analyte through the tissue. Therefore the model with easily accessible and intact tissues could not be applied. The maximum yields of lignans obtained at 50 °C and 27 MPa are listed in Table 2. No estimate of the residual amount of analyte in the matrix could be made from these experiments.

More details on the SC-CO_2 extraction from *S. chinensis* caulomas and leaves will be given elsewhere [13].

5.3. Extraction with ethanol

The extraction yields of lignans obtained with ethanol are listed in Table 2. It is evident that ethanol was less efficient than SC-CO_2 in the case of caulomas, where the ratio of total lignan yield obtained with SC-CO_2 to that obtained with ethanol was 2.1. In contrast, the ratio of total lignan yield obtained from leaves with SC-CO_2 to that obtained with ethanol was only 0.88. Lignan yields in etha-

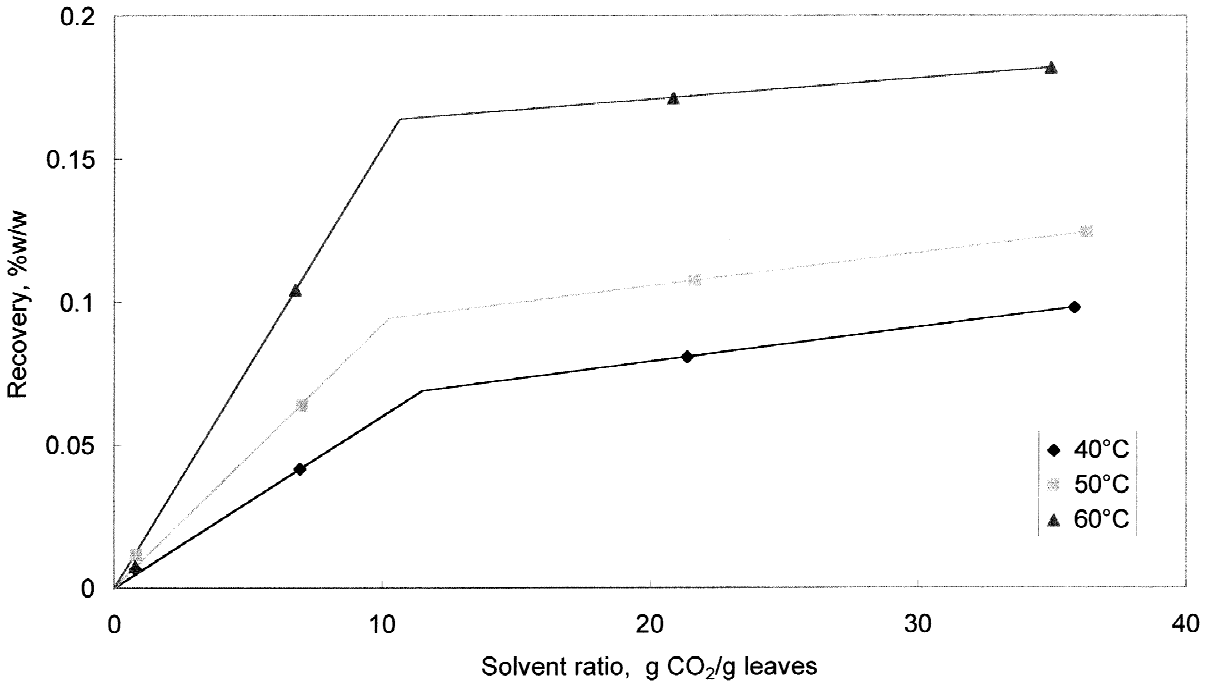

Fig. 4. Supercritical fluid extraction of cinnamic acid from leaves (27 MPa, 0.9 g min^{-1} CO_2).

nolic extract from leaves were higher in the SC-CO_2 extracts, except for schisandrin. This confirms the previous conclusion that supercritical fluid extraction of lignans from leaves was not complete due to their interaction with plant matrix.

6. Conclusion

The described HPLC separation in reversed-phase mode with UV detection is a very reliable analytical procedure for determination of lignans and similar compounds with a relatively large lipophilic molecule. It is especially suitable when the analysis is not limited by small amount of sample.

The SC-CO_2 extraction of lignans from caulomas was efficient and the process was described using a model distinguishing easily accessible and intact vegetable tissues. The SC-CO_2 extraction of lignans and cinnamic acid from leaves was not complete because of the strong interaction of analytes with plant matrix.

Acknowledgements

The authors thank Petr Stodůlka for the HPLC consultation and performing part of the HPLC analysis, and M. Koptová for preparation of the extract samples. The project is carried out within the grant no. 203/01/0550, Grant Agency of the Czech Republic.

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