Chemistry of Natural Compounds, Vol. 39, No. 2, 2003

AN IMPROVED ON-LINE HPLC-DPPH* METHOD FOR THE SCREENING OF FREE RADICAL SCAVENGING COMPOUNDS IN WATER EXTRACTS OF LAMIACEAE PLANTS

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UDC 547.913

An on-line HPLC-1,1-diphenyl-2-picrylhydrazyl (DPPH) method has been improved for the detection of polar and nonpolar radical scavenging compounds from complex plant extracts. Eight water extracts were prepared from steam-distilled essential oil-extracted Lamiaceae plants (*Origanum vulgare *L.*, O. onites *L.*, O. minutiflorum *O. Schwartz et P. H. Davis*, O. syriacum *L.*, Satureja cuneifolia *Ten.*, Thymbra spicata *L.*, Coridothymus capitatus *(L.)* Reichb. f., Majorana hortensis *Moench). After the components within each extract had been separated by reverse phase chromatography using 10% to 100% methanol with 2% acetic acid as a mobile phase, analytes capable of scavenging a citric acid-sodium citrate buffered methanolic DPPH* solution were detected by post-column derivatization at 517 nm. The HPLC-DPPH* on-line method was applied to the qualitative and quantitative analysis of these Lamiaceae plant extracts. There was a strong correlation between the scavenging (negative) peak area and the concentration of the radical scavenging reference substances used. The radical scavenging compounds within the extracts were determined as benzoic acid and hydroxycinnamic acid derivatives, flavonoids and diterpenoids according to their retention time and UV spectral data. Rosmarinic acid and carnosic acid were identified as the dominant radical scavengers in these extracts by this method.*

Key words: Lamiaceae; online HPLC-DPPH*; antioxidants; radical scavenging compounds; 1,1-diphenyl-2-picrylhydrazyl (DPPH*).

Oxygen is the most important element for aerobic life, however, it may also participate in a number of toxic chemical reactions. The chemical reaction that usually takes place between atmospheric oxygen and an organic compound is generally defined as autoxidation. Autoxidation can affect foodstuffs that contain lipids [1, 2]. Lipid peroxidation is the principal cause of the organoleptic deterioration of foodstuffs during processing, distribution, and storage. Thus, the protection of foods against such deterioration is of great economic and nutritional importance to the food industry [3, 4]. Therefore, antioxidants may be considered an important tool to protect susceptible products from oxidative deterioration [5]. Furthermore, oxidized polyunsaturated fatty acids (PUFA) may induce premature aging and carcinogenesis [6].

Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and t-butyl hydroquinone (TBHQ) have commonly been used to extend the shelf life of foodstuffs by inhibiting and/or delaying the onset of the oxidation process [7–10]. However, the possible toxicity of synthetic antioxidants has resulted in decreased use of these compounds in foods for human consumption [7]. As a consequence of this and due to the appeal of natural products to consumers, numerous studies have been carried out in order to identify naturally occurring compounds which possess antioxidant activities such as phenolic phytochemicals [4, 11, 12].

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0009-3130/03/3902-0161$25.00

TABLE 1. Extract Yield and HPLC Qualitative and Quantitative Data for *O. vulgare* and By-product Extracts

Components	Quantification (HPLC)[a]							
	Origanum vulgare	*Coridothymus capitatus*	*Majorana hortensis*	*Origanum minutiflorum*	*Origanum onites*	*Origanum syriacum*	*Satureja cuneifolia*	*Thymbra spicata*
Identified components								
EY[b]	48.4	25.2	24.2	29.0	30.4	29.0	31.8	29.2
Caffeic acid (13.9)[c]	0.54±0.04	0.12±0.01	0.12±0.00	0.14±0.00	0.12±0.02	0.21±0.02	0.07±0.00	0.13±0.01
Rosmarinic acid (32.4)	13.25±1.03	6.37±0.36	5.09±0.59	6.36±0.01	3.96±0.53	7.84±0.07	5.47±0.22	6.75±0.32
Apigenin (42.1)	0.30±0.03	0.01±0.01	0.42±0.04	0.10±0.01	0.15±0.01	0.19±0.01	0.29±0.03	0.15±0.02
Luteolin-7-O-glucoside (26.2)	2.93±0.39	1.37±0.00	2.58±0.24	1.70±0.14	Tr.	4.03±0.40	1.04±0.10	1.84±0.14
Naringin (27.6)	n.d.	n.d.	n.d.	n.d.	n.d.	0.23±0.00	0.30±0.01	n.d.
Unidentified components								
Σ Benzoates[d]	20.14±0.08	0.95±0.05	0.64±0.04	0.57±0.21	7.49±0.09	1.24±0.01	1.12±0.91	4.18±0.20
Σ Hydroxycinnamates[e]	0.61±0.03	0.73±0.01	0.87±0.01	1.03±0.03	0.41±0.02	2.46±0.00	1.12±0.01	0.34±0.02
Σ Flavonoids[f]	10.87±0.14	2.46±0.04	4.26±0.02	6.87±0.05	3.45±0.04	4.32±0.03	3.03±0.03	4.99±0.06
Σ total	48.64±1.12	12.01±0.37	13.98±0.64	16.77±0.26	15.58±0.54	20.52±0.41	12.44±0.94	18.38±0.41

[a]: mg/g (dry weight) by-product; [b]: EY, extract yield is expressed as %, w/w; [c]: Retention time (min); [d]: Quantitated using *p*-hydroxybenzoic acid; [e]:Quantitated using caffeic acid; [f]: Quantitated using apigenin. n.d., Not detected.
Tr.: Trace. Values are presented as mean ± standard error of the mean for triplicate analyses.

In recent years, phytochemicals such as ascorbic acid and a-tocopherol and crude herb and spice-derived extracts have appeared on the market and are generally regarded as safe (GRAS), naturally occurring antioxidants for food industry use. The botanical family Lamiaceae includes a large number of plant species that are well known for their antioxidant-rich properties. Members such as rosemary *(Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), and oregano (*Origanum vulgare* L.) have been widely studied and the majority of their antioxidant components (rosmarinic acid, carnosic acid, carnosol, etc.) have been isolated and identified [13].

Activity-guided fractionation of plant extracts is a time-consuming, labor-intensive, and expensive process, and loss of activity during the isolation and purification procedures is very common due to dilution effects or decomposition [14, 15]. Furthermore, successful fractionation may only identify presently characterized compounds and not new ones [14]. For this reason, the availability of a rapid and cost-effective method for screening and activity evaluation of the samples is essential in order to avoid many of the above-mentioned problems. A method combining separation and activity evaluation would present a major advantage for such investigations. However, reports concerning on-line separation and antioxidant activity assessment are scarce.

HPLC-coupled activity reactions include chemiluminescent reactions [16, 17], and reactions with stable nitrogen-centered free radical species such as 1,1-diphenyl-2-picrylhydrazyl (DPPH*) [15–20] or 2,2′-azobis-(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\cdot+}$) [21] have been used for on-line separation and antioxidant activity tests. However, the HPLC-DPPH* on-line activity test is more widely used than the other techniques. This is because this free radical is the most widely used radical for determining free radical scavenging properties of potential antioxidants and the available DPPH* scavenging tests are described as simple and useful in the screening of large numbers of compounds/extracts, and are convenient [22].

The aim of the present article is to determine radical scavenging compounds in water extracts of some Lamiaceae plants, based on a post-column reaction of the antioxidant with the DPPH* radical. In addition, these active phenols were analyzed quantitatively using the HPLC-UV method.

TABLE 2. Minimum Detectable Concentration (MDC) and Minimum Detectable Amount (MDA) of Phenolic Compounds in Water Extracts of Lamiaceae plants in the On-line HPLC-DPPH System

Standards	t_R (min)	MDC (mg/mL)	MDA (ng)	Calibration equations	Calibration coefficient (r^2)
Caffeic acid	13.9	0.72	18.11	$y = 2.10\times10^8x$ - 144884	0.999
Luteolin-7-O-glucoside	26.2	0.43	10.64	$y = 7.10\times10^7x$ - 29828	0.999
Naringin	27.6	1.17	29.26	$y = 5.10\times10^7$ - 58543	0.999
Rosmarinic acid	32.4	0.02	0.50	$y = 5.10\times10^7$ - 1028	0.999
Apigenin	42.1	0.27	6.84	$y = 7.10\times10^7$ - 19180	0.999

In this study, chromatographic separations, post-column radical scavenging activities, and quantitative analysis of water extracts were carried out by the on-line HPLC-DPPH· method. In this study, plant materials from which essential oils had previously been removed were used. Essential oil-free plant materials were extracted with water, and eight water extracts were obtained. The yields of the extracts are given in Table 1. All these extracts were separated on the reverse phase HPLC column using an acidic methanol–water solvent system and detected with a PDA detector. After UV detection, the analytes were derivatized with buffered methanolic DPPH* solution and the radical scavengers within the extracts were detected at 517 nm. Simultaneously obtained UV and DPPH* quenching chromatograms under gradient conditions of eight different water extracts from Lamiaceae plants are presented in Fig. 1. Reference phenols within the extracts were also analyzed by on-line HPLC-DPPH* methods either qualitatively or quantitatively. Caffeic acid, rosmarinic acid, luteolin-7-O-glucoside, naringin, and apigenin in the extracts were analyzed quantitatively and the amounts of phenols in the extracts are given in Table 1.

For determination of the limits of detection of the tested compounds ($LD_{compound}$, mg/mL), the Student's t-statistic $t_{0.005} = 2.07$ for n =15 measurements of the blank signal with a confidence interval of 95% was used. The negative peak resulting from the bleaching of DPPH by a radical scavenging compound is considered detectable if its height exceeds the calculated $LD_{compound}$. The MDC and MDA of compounds were calculated using calibration curves and are shown in Table 2.

Based on the latest literature, the family Lamiaceae seems to be a rich source of plant species containing large amounts of phenolic acids, especially as depsides such as rosmarinic acid, flavones, and their glycosides [23, 24]. Based on the qualitative and quantitative results, rosmarinic acid is the main phenolic acid dimer in plants. For identification of phenolics in the by-product and *O. vulgare* extracts, a high performance liquid chromatography-diode array detection technique was used. The identification of known compounds (caffeic acid, luteolin-7-O-glucoside, rosmarinic acid, naringin, and apigenin) in the extracts was carried out by comparing their t_R values and UV spectra with those of standards. Quantitative data for these compounds were calculated from their respective calibration curves. Unknown compounds were identified to the level of phytochemical class (e.g., benzoate, hydroxycinnamate, flavonoid) from their recorded spectral data.

Quantitative data for compounds identified as either benzoates, hydroxycinnamates, and flavonoids were calculated by using 4-hydroxybenzoic acid, caffeic acid and apigenin calibration curves, respectively. Based on the HPLC results, *O. vulgare* contain higher amounts of phenolics (65.66 mg/g) than the other by-products, and rosmarinic acid was found to be the main compound (2.98–13.25 mg/g) in all extracts. In addition, all water extracts from the selected plant material were found to be rich in flavonoids in their glycosidic form (2.40–10.87 mg/g).

The importance of being able to identify which components within a complex mixture are responsible for the activity of the mixture as a whole is obvious. Traditional pharmacognostic procedures used to answer such issues are characteristically long, labor-intensive, expensive, and may only result in the isolation and identification of known substances. Any technology which can be used for dereplication as early as possible clearly has enormous benefit. The technology presented in this communication can be used for such proposes. From the chromatogram and combined activity profile, sufficient data are available to identify known compounds or the chemical class of unknowns, identify which components possess radical scavenging activity, and, from the observed chromatographic behavior, gain important information about conditions required for the isolation of new analytes with such important activity.

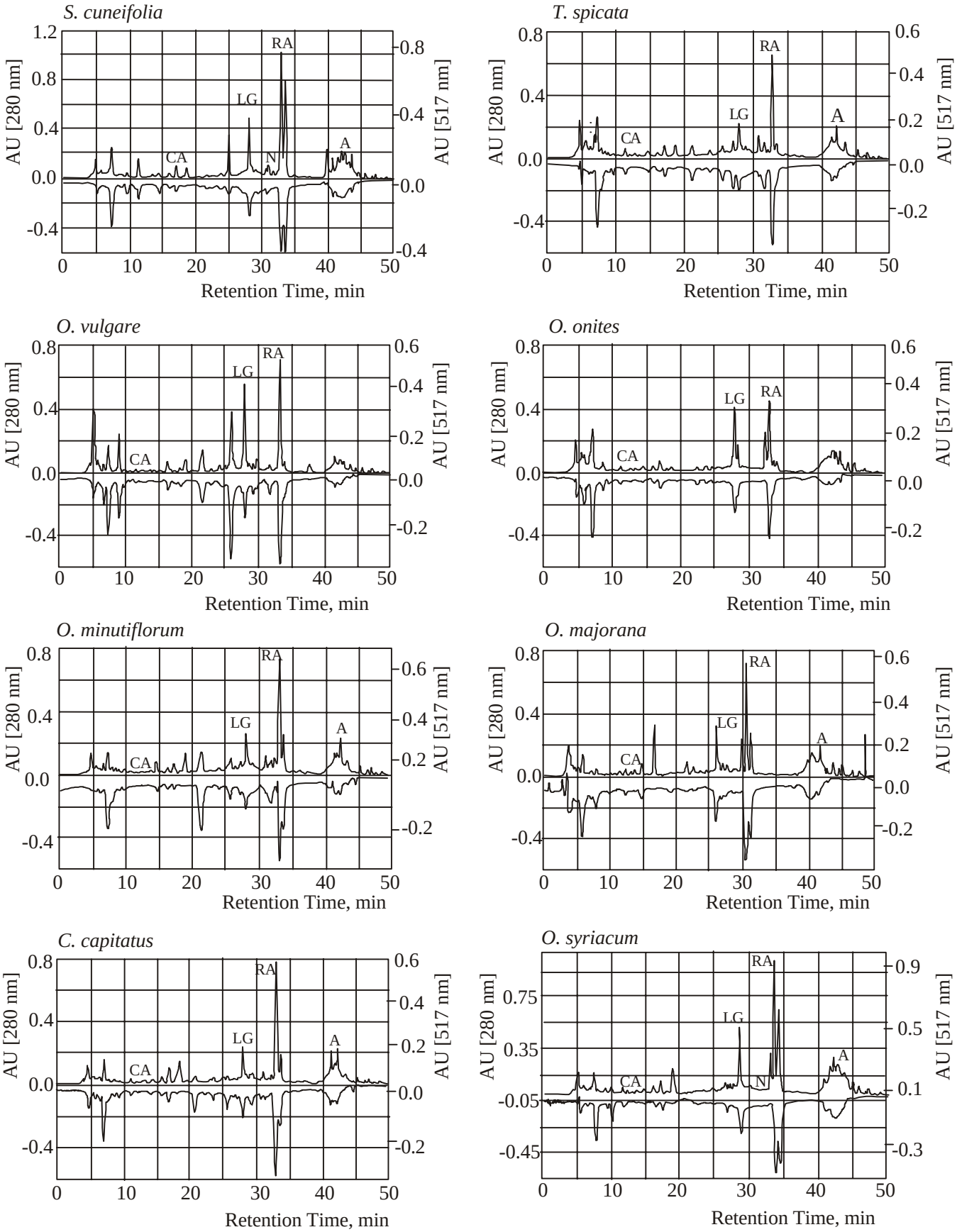

Fig. 1. Uv and DPPH radical quenching chromatograms of water extract [caffeic acid (CA); luteolin-7-*O*-glucoside (LG); naringin (N); rosmarinic acid (RA); apigenin (A)].

EXPERIMENTAL

Plant Material and Reagents. Essential oil-free Spanish oregano (*Coridothymus capitatus* (L.) Reichb. f.; syn. *Thymbra capitata* (L.) Cav.; *Thymus capitatus* (L.) Hoffm. et Link, sweet marjoram (*Majorana hortensis* Moench.; syn. *Origanum majorana* L.), Toka oregano (*Origanum minutiflorum* Schwarz et Davis), Turkish oregano (*Origanum onites* L.), Syrian oregano (*Origanum syriacum* L.; syn. *Majorana syriaca* L.), savory (*Satureja cuneifolia* Ten.), black thyme (*Thymbra spicata* L.), and an oregano ground mixture (subsequently referred to as ground mix) were obtained from Turer Ltd. (Izmir, Turkey). Greek oregano herb (*Origanum vulgare* L.) was obtained from Pimenta Oy, Finland. Ultrapure water (18.2 MΩcm, HPLC grade) was prepared by a Millipore Milli-RO 12 plus system (Millipore Corp., Bedford, Massachusetts). All solvents were of analytical grade and purchased from the usual sources. 1,1-Diphenyl-2-picrylhydrazyl (95%) was obtained from Aldrich (Steinheim, Germany), rosmarinic acid, apigenin, naringin, and luteolin-7-O-glucoside from Extrasynthese (Genay, France), and caffeic acid from Sigma (Steinheim, Germany).

Extraction Procedure. Plant material was sieved and 50 g was transferred into a 1 L round-bottomed flask to which was added 600 mL ultrapure H_2O. The plant material was hydrodistilled for a period of 2 h using a Eur. Ph. hydrodistillation apparatus. The remaining marc was hydrodistilled a further 2 times with 400 mL H_2O. This was done in order to ensure the absence of essential oil in the final extract. The combined aqueous extracts were filtered to remove the plant material, reduced in volume in vacuum at 45°C, freeze-dried, and stored at 4°C.

High Performace Liquid Chromatography (HPLC) Set-up. The liquid chromatographic apparatus (Waters 600) consisted of an in-line degasser, pump, and controller coupled to a 2996 photodiode array detector equipped with a Rheodyne injector (20 µL sample loop) interfaced to a PC running Millenium^{32} chromatography manager software (Waters Corp., Milford, Massachusetts). Separations were performed on a reverse-phase Hypersil BDS-C18 analytical column (250 x 4.6 mm i.d., particle size 5 µm) (Agilent Technologies, Milford, Massachusetts) operating at room temperature with a flow rate of 0.7 mL/min. Detection was carried out with a sensitivity of 0.1 a.u.f.s. between the wavelengths of 200 to 550 nm. Elution was effected using a ternary nonlinear gradient (Table 1) of the solvent mixture MeOH–H_2O–CH_3COOH (10:88:2, v/v/v) (solvent A), MeOH–H_2O–CH_3COOH (90:8:2, v/v/v) (solvent B), and MeOH (solvent C). The components were identified by comparison of their retention times to those of authentic standards under analysis conditions, and the UV spectra with our in-house PDA library. A 10 min equilibrium time was allowed between injections.

HPLC Post-column Derivatization. On-line post-column addition of DPPH*reagent was performed using a Waters 515 HPLC pump. DPPH* radical scavenging detection was carried out at 517 nm with a sensitivity of 0.05 a.u.f.s. using a 2487 Dual λ Absorbance UV-Vis detector (Waters Corp., Milford, Massachusetts). A 13 m long reaction coil (0.25 mm i.d.) PEEK tubing (Waters Corp., Milford, Massachusetts) was interfaced between the UV detector and DPPH* reagent pump via a T-junction. The DPPH* reagent was prepared in methanol at the beginning of each day of analysis at a concentration of 70 mg/mL and kept protected from light. This solution was mixed with citrate buffer at a ratio of 3:1. The buffer, pH 7.6, contained 0.05 M citric acid and 0.05 M sodium citrate solutions. This DPPH* reagent was filtered through a 0.45 µm membrane filter and degassed before use. The flow of DPPH* reagent was 0.7 mL/min.

Detection Limits, Minimum Detectable Amounts and Minimum Detectable Concentrations. Detection limits (LD, arbitrary units) were calculated using Eq (1), as described by Koleva et al. [17], where *t* is the Student's *t*-statistic and $\sigma_{\text{blank signal}}$ is the standard deviation of the blank signal (n = 15).

$$LD = -2t\sigma_{\text{blank signal}} \quad (1)$$

The minimum detectable concentration (MDC) values in µg/mL units were calculated for each compound by using the calibration equations. These equations were used to calculate the MDC using the previously calculated LD value as y. Minimum detectable amounts in ng units were calculated from the MDC values taking into account the injection volume used.

Quantitative Analysis. All extracts were dissolved in 70% (aq.) methanol at a concentration of 10 mg/mL. For the stock solution of the standards, the polyphenols (caffeic acid, luteolin-7-O-glucoside, naringin, apigenin, and rosmarinic acid) were dissolved in 70% (aq.) methanol at a concentration of 1 mg/mL. The concentration ranges of reference compounds used for calibration of the HPLC analysis were 0.01–0.10 mg/mL. All samples and standards were injected three times.

ACKNOWLDGEMENT

The authors wish to acknowledge the financial support of the Paulig Group Ltd., Finland.

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