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Short Communication

Multichannel coulometric detection coupled with liquid chromatography for determination of phenolic esters in honey

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ABSTRACT

A method for the determination of phenolic esters in different varieties of honey was developed. The substances were separated by RP-HPLC. A coulometric electrode-array system with sixteen electrodes arranged in series and set at increasing potentials (300–900 mV) was used for electrochemical detection of the compounds. Chromatographic peaks for methyl 4-hydroxybenzoate, methyl vanillate, methyl syringate, *trans*-*p*-methyl coumarate and *trans*-methyl ferulate were identified. The content of the esters varied between 1.3 and 5044 μg per kg of honey with detection limits of 0.1-1.0 μg per kg of honey (SIN = 3). The method described is a sensitive assay to differentiate between rape honey and other varieties.

INTRODUCTION

Honey is a well-known and popular foodstuff. Because consumers attach more and more importance to high quality and great variety, reliable methods for its analysis are required. Attention has been focused on investigations to find ingredients that characterize individual types of honey. Among them are phenolic acids [1–11], which occur in trace amounts. Esters of phenolic acids have also been found [5,7,12,13]. Up to now mainly GC and GC-MS methods have been employed for the determination of these compounds in honey.

Since most phenolic compounds are oxidized at easily accessible potentials, electrochemical detection can serve as a highly sensitive tool for their quantification after separation by HPLC. As yet it has only been applied to the determination of the distribution pattern of phenolic acids in different varieties of honey [10,11]. These investigations distinguished between blossom honey, honeydew honey and chestnut honey, but it was not possible to differentiate between rape and robinia honey, two types of blossom honey. The objective of the research described in this paper was the determination of phenolic esters in different varieties of honey using reversed-phase chromatography coupled with a coulometric electrode-array detection system.

EXPERIMENTAL

Apparatus

For single-cell detection a Gynkotek pump

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(Model 300C) was coupled with a Rheodyne injection valve (Model 7125, sample loop: 100 μl), a Bio-Rad RoSiL C_{18} HL (25 cm × 4.6 mm I.D., particle size 5 μm) column and an ESA analytical cell (Model 5011). An ESA coulochem 5100 was used as the controlling and measuring unit. The response of the detection cell was recorded by a Sekonic SS-250-F recorder (Fig. 1). For all the other chromatographic experiments an ESA coulochem electrode-array system was employed, consisting of two HPLC pumps (Model 420) and a multichannel detector with sixteen coulometric electrodes and their electronic control and measuring unit. Chromatographic separation was performed on a Bio-Rad RoSiL C_{18} HL column (25 cm × 4.6 mm I.D., particle size 5 μm). A Rheodyne valve (Model 7125) with a 100-μl sample loop was used for sample injection. Each of the sixteen detector cells consisted of one working electrode made of porous graphite, two platinum counterelectrodes and two reference electrodes of modified palladium. For data processing an Epson Equity 386/25 computer with the appropriate software from ESA was used. The results and the sixteen-channel chromatograms were printed on an Okidata microline 321 printer.

Mobile phase

All chemicals used and not specified were from Merck and of p.a. quality. The mobile phase consisted of 300 ml of methanol, 50 ml of glacial acetic acid and 650 ml of double-distilled water. The pH of the solution was adjusted to 3.0 with an aqueous solution of sodium hydroxide (1 *M*). The mobile phase was filtered through a 0.20-μm membrane.

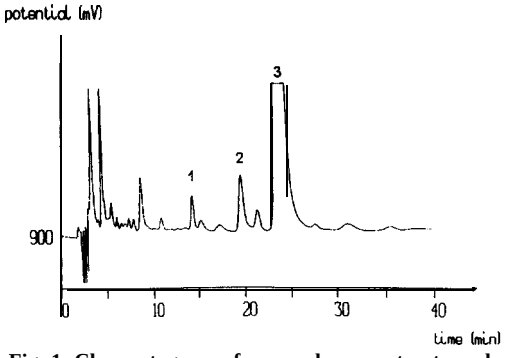

Fig. 1. Chromatogram of a rape honey extract produced by a single detector cell. Full-scale current amounts to 500 nA. Peaks: 1 = methyl 4-hydroxybenzoate; 2 = methyl vanillate; 3 = methyl syringate.

Standards

Stock solutions with a concentration of 25 mg/l methyl 4-hydroxybenzoate, methyl 4-hydroxy-3-methoxybenzoate (methyl vanillate), methyl 4-hydroxy-3,5-dimethoxybenzoate (methyl syringate), *trans*-methyl 4-hydroxycinnamate (*trans*-*p*-methyl coumarate) and *trans*-methyl 4-hydroxy-3-methoxycinnamate (*trans*-methyl ferulate) were prepared separately in methanol-water (10:90). All chemicals were taken from our own stock. The standard solutions were mixed and further diluted with mobile phase to concentrations between 0.1 μg/l and 1 mg/l. All calibration mixtures were stored in a refrigerator and kept in the dark to prevent light-induced *cis–trans* isomerization.

Chromatographic conditions

The elution of the esters was effected by a flow-rate of 1 ml/min, which resulted in a back-pressure of approximately 180 bar. For single-cell detection a potential of +900 mV was chosen. The sixteen electrodes were set at incrementally (40 mV) increasing potentials, covering a potential range between +300 mV and +900 mV. Temperature in the thermally insulated cell box containing the column, pulse damper, injection loop and coulometric cells was held constant between 31.6 and 31.7°C.

Samples

The types of honey under investigation were commercially available products of chestnut, clover, dandelion, fir, linden blossom, orange blossom, rape, robinia (false acacia) and sunflower honey. All samples were kept in the dark.

Sample preparation

In a beaker 20 g of honey and 18 ml of ethyl acetate (>99%, Fluka) were mixed with a high-speed dispersing apparatus (Ultra-Turrax T-25) and the ethyl acetate phase was decanted. This procedure was repeated three times and all extracts were collected. Then the honey sample

was mixed with diluted hydrochloric acid (4 *M*) until a pH between 1 and 2 was reached. Extraction with 18 ml of ethyl acetate was repeated six times. During the whole procedure the beaker was cooled on ice. The ethyl acetate extracts were combined and concentrated to a volume of about 10 ml using a rotary evaporator and dried over anhydrous sodium sulphate. This procedure was followed by extraction (four times) with 10 ml of sodium hydrogencarbonate solution (5%, w/w) to remove phenolic acids. After drying the ethyl acetate phase over sodium sulphate this solution was evaporated to dryness. The residue was dissolved in 10 ml (in the case of robinia, chestnut, sunflower and orange blossom honey) or in 100 ml of mobile phase (rape, clover, linden blossom, dandelion and fir honey). The solutions were filtered through a 0.2-μm membrane.

Synthetic honey

A mixture of 400 g of D(+)-glucose (Merck) and 400 g of fructose (Apoka) with 200 ml of double-distilled water was prepared. The pH was adjusted to 4.5 with dilute hydrochloric acid (0.01 M). Aliquots of 20 g were mixed with 2 ml of the calibration mixture containing all esters in a concentration 0.75 mg/l.

Data analysis

Each substance showing a definite electrochemical behaviour was detected on several adjacent electrodes (channels). Plotting the peak heights (areas) of the substances of interest against the potentials of the channels, peak-shaped hydrodynamic voltammograms were obtained. Chromatographic peaks were identified by retention times as well as the hydrodynamic voltammograms. Peak purity was tested in the following way. The current of the dominant peak (highest response) and the corresponding peaks with the same retention time shown on adjacent channels with lower and higher potentials were measured. The height ratios of the adjacent peaks to the dominant one were calculated. These ratios were compared with the ratios of the standard compounds calculated in the same way. The esters with shorter retention times were quantified by measurement of the recorded chromatographic peak heights. The results of later-eluting esters relied on peak areas. Calibration curves were created each day in a concentration range between 10 and 100 μg/l. The recovery of the overall procedure was determined by analysing three samples of synthetic honey.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram of a rape honey extract, which was obtained by single-cell detection at a potential of +900 mV. A preliminary identification of the chromatographic peaks was done by comparing retention times of standard compounds and sample components. However, this detection mode gives only little information about peak purity and allows no reliable peak identification.

Coulometric multielectrode detection and qualitative analysis

The current measured at sixteen electrodes held at different potentials led to several chromatographic peaks for each substance of the honey extracts (Fig. 2). Peak purity could be

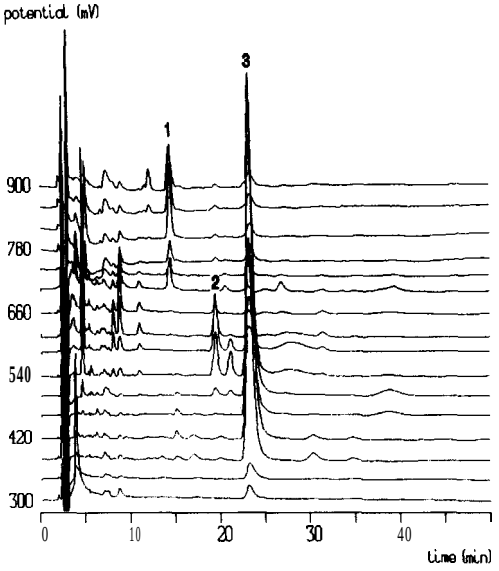

Fig. 2. Chromatograms of a rape honey extract produced by sixteen detector cells. Full-scale current amounts to 500 nA. Peaks: 1 = methyl 4-hydroxybenzoate; 2 = methyl vanillate; 3 = methyl syringate.

TABLE I

CHARACTERISTIC VALUES OF METHYL 4-HYDROXYBENZOATE, METHYL VANILLATE AND METHYL SYRINGATE

	Methyl 4-hydroxybenzoate		Methyl vanillate		Methyl syringate	
	Standard	Honey	Standard	Honey	Standard	Honey
Retention time (min)	14.37	14.38	19.38	19.38	22.28	23.23
Channel of the dominant peak (mV)	+820	+ 820	+580	+580	+420	+ 420
Peak-height ratio						
h_{x-1}/h_x	0.305	0.305	0.785	0.772	0.643	0.606
h_{x+1}/h_x	0.803	0.948	0.589	0.602	0.760	0.851

confirmed by calculating the peak-height ratios of the preceding (h_{x-1}) and the following peaks to the dominant peaks (h_x). Table I shows a list of these values. Deviation of the peak-height ratios of sample components from those of standard compounds is due to co-eluted substances. In contrast to single-cell detection, voltammetric characterization of the eluted substances (Fig. 3) could be achieved during one LC-electrochemical detection experiment by plotting the peak heights at definite retention times *versus* the applied potentials of the electrodes (channels). The electrode (channel) potential at which the highest response (dominant peak) is observed corresponds almost exactly to the half-wave potential of the compound. The initial identity assignment by retention times could be confirmed by comparing the channels of the dominant peaks and the shape of the hydrodynamic voltammograms of standard and sample compounds. In this way methyl 4-hydroxybenzoate, methyl vanillate and methyl syringate were identified in rape honey.

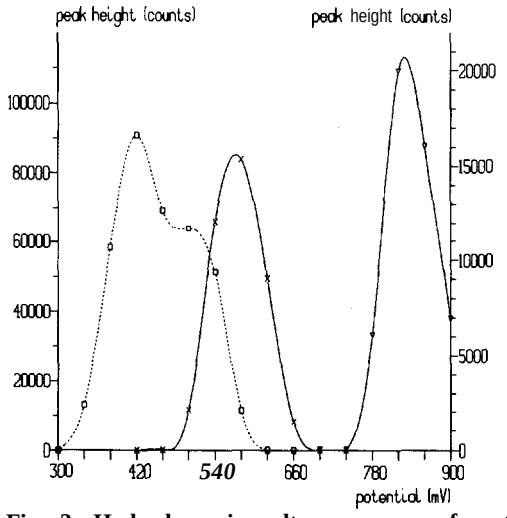

Fig. 3. Hydrodynamic voltammograms of methyl 4-hydroxybenzoate (V), methyl vanillate (X) and methyl syringate (Cl) plotted with different sensitivity (left-hand axis: Cl; right-hand axis: X and V).

Quantitative analysis

To three samples of synthetic honey methyl 4-hydroxybenzoate, methyl vanillate, methyl syringate, *trans-p*-methyl coumarate and *trans*-methyl ferulate were added and analysed. The recovery obtained ranged between 65.2 and 79.4% with relative standard deviations between 1.5 and 6.7%.

All these compounds were found in different varieties of honey. In Table II the mean values of three samples of each variety of honey except rape and robinia honey are summarized. The detection limits ranged between 0.1 and 1.0 μg per kg honey (20-200 pg absolute) depending on the retention times of the esters.

Comparing the results of the esters, significant differences between types of honey could be observed. In *robinia honey* only methyl syringate was found, as can be seen in Fig. 4. Methyl 4-hydroxybenzoate and methyl vanillate were near or below the detection limits. *Rape honey* was characterized by its very high concentration of methyl syringate. The amount of this ester in

TABLE II

CONTENT (fig/kg) OF PHENOLIC ESTERS IN HONEY

M4HB = Methyl 4-hydroxybenzoate; MVAN = methyl vanillate; MSYR = methyl syringate; MCOM = *trans-p*-methyl coumarate; MFER = *trans*-methyl ferulate

Variety	M4HB	MVAN	MSYR	MCOM	MFER
Robinia	–		281.0	–	–
	–	3.5	93.4	–	
Rape	177.0	31.3	3865	–	–
	126.2	36.7	4092	–	–
	184.8	237.4	4537	–	
	167.3	35.2	5044		–
Chestnut	18.0	10.9	42.2	–	–
Clover	38.3	11.8	1592	–	–
Linden blossom	7.7	20.2	759.5	–	–
Dandelion		11.8	551.5		1.3
Sunflower	31.8	8.0	322.7	1.5	2.9
Orange blossom	194.2	18.8	–	–	7.9
Fir	–	14.7	539.5	–	1196

various samples of robinia and rape honey varies naturally, but this did not influence the significant differences. In addition, the content of methyl 4-hydroxybenzoate was higher than in other types except in orange blossom honey. Some small differences were observed between the varieties *chestnut, clover, dandelion, linden blossom,* and *sunflower,* but in this case the results were too few to .be significant. The absence of methyl syringate was characteristic of *orange blossom honey,* which also had the highest content of methyl 4-hydroxybenzoate of the varieties analysed. *Fir honey,* the only honeydew honey under investigation, contained a similarly large amount of ***trans*-methyl** ferulate, whereas this ester was not present or occurred in very low concentration in other types of honey. This made an identification possible.

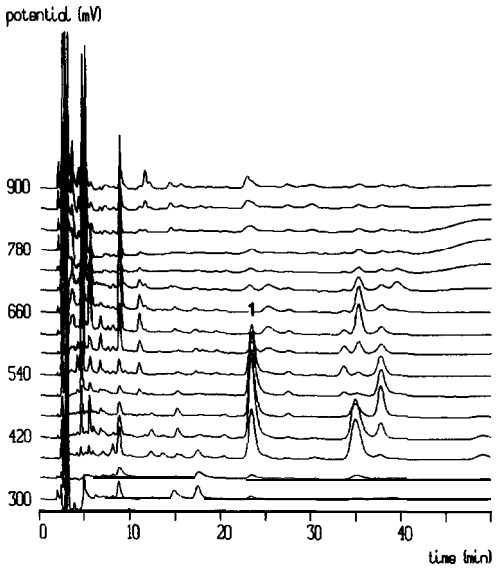

Fig. 4. Chromatograms of a robinia honey extract produced by sixteen detector cells. Full-scale current amounts to 1.5 μA. Peak 1 = methyl syringate.

The results obtained for methyl esters **confirm** and complement information received from preliminary investigations of phenolic acids in honey **[11].** They allowed differentiation between rape honey and robinia honey, which was not possible by analysis of the phenolic acids alone.

CONCLUSION

The coulometric **multielectrode** detection system provides the trace analyst with another tool for reliable determination of electroactive compounds. This technique can be used **to** extend the qualitative information without the time-consuming current-potential measurements usually necessary when single-electrode detection is used. A particular advantage is that the optimal detection potential for a compound of

interest is lower than for the detection of the same compound in a single cell. This results in a lower residual current and makes possible very sensitive detection of phenolic esters. In subsequent investigations the selectivity of this detection mode will be evaluated by analysing samples of environmental importance and food.

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