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Simultaneous liquid chromatographic analysis of 5-(hydroxymethyl)-2-furaldehyde and methyl anthranilate in honey

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A method for the simultaneous determination of 5-(hydroxymethyl)-2-furaldehyde and methyl anthranilate in honey is reported. The analysis is carried out by reversed-phase gradient-elution high performance liquid chromatography (HPLC). The proposed method can be applied for both the routine characterization of honey based on the methyl anthranilate content and the quality control analysis based on the 5-(hydroxymethyl)-2-furaldehyde level.

INTRODUCTION

Low levels of 5-(hydroxymethyl)-2-furaldehyde (hydroxymethyl-furfural, HMF) are usually detected in honey due to the degradation of sugars catalysed by the normal honey acidity at room temperature. However, this degradation is accelerated during heat processing or storage at high temperatures. The occurrence of high levels of HMF in honey may also be caused by adulteration with commercial invert syrup (White & Siciliano, 1980). Thus, HMF content in honey allows an evaluation of the honey quality. The Codex Alimentarius Commission Standards (1981) included a maximum value for HMF in table honey of 4 mg/100 g. Hence, rapid and accurate methods for determining the HMF content to discriminate between genuine honey abused by heat or storage and adulterated honey are required. Proposed procedures have applied spectrophotometric (Dhar & Roy, 1972; White, 1979*a,b*; White *et al.*, 1979; White & Siciliano, 1980; Wootton & Ryall, 1985; Lord *et al.*, 1989) or chromatographic (Jeuring & Kuppers, 1980; Marini & Righi, 1985; Wootton & Ryall, 1985) techniques.

Methyl anthranilate (MA) is contained in *Citrus* honeys giving a distinctive and pleasant flavour to the product. Thus, the MA concentration in the honey allows the differentiation between monofloral *Citrus* honeys and other monofloral or multifloral honeys (Deshusses & Gabbai, 1962; Dörrscheidt & Friedrich, 1962; White *et al.*, 1962; Hoopen, 1963; Merz, 1963; White *et al.*, 1964; Cremer & Reidmann, 1965; White, 1965; Chogovadze *et al.*, 1973; Wootton *et al.*, 1978; Graddon *et al.*, 1979; Bicchi *et al.*, 1983; Serra, 1988). All proposed methods for MA determination included spectrophotometric or chromatographic techniques.

No studies concerning the simultaneous determination of MA and HMF in honey have been reported. In the present study, a fast and simple reversed-phase high performance liquid chromatography (HPLC) procedure for the simultaneous analysis of these compounds in honey is described. The method can be applied to the routine characterization of honey based on the methyl anthranilate content as well as to quality control analysis of HMF to evidence improper processing and storage or adulteration by invert syrup.

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MATERIALS AND METHODS

Apparatus

The HPLC system consisted of a Perkin-Elmer Series 4 liquid chromatograph operating at room temperature and with a flow-rate of 1 ml/min; a Perkin-Elmer LC-85B variable wavelength scanning spectrophotometric detector with a 8-μl flow-cell and wavelength set at 283 nm for HMF and 337 nm for MA, a 7125-075 Rheodyne injector valve with a 20-μl sample loop and a Perkin-Elmer Sigma 15 chromatography data station. The column was 12·5 × 0·46 cm i.d. stainless steel packed with Spherisorb ODS-2 with a particle size of 5 μm. A Supelco guard column packed with the same stationary phase was also used.

Reagents

Methanol, acetonitrile, methylene chloride and ethyl acetate (Romil Chemicals, Loughborough, Leicester, UK) were liquid chromatographic grade. Doubly distilled water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). The solvents were degassed by sparging them with helium gas.

5-(Hydroxymethyl)-2-furaldehyde (HMF) and methyl anthranilate (MA) were obtained from Sigma (St. Louis, MO, USA). Solutions were prepared from the commercial products, without further purification, by dissolving 50 mg in 50 ml of methanol and stored in dark bottles in the cold. Working solutions were obtained by dilution with methanol immediately before the measurements and kept in the dark to avoid decomposition of HMF by light. The concentration of standard HMF was confirmed spectrophotometrically by using the UV molar absorptivity (Turner *et al.*, 1954).

Calibration graphs

The separation requires a gradient elution technique using a binary system: the initial chromatographic conditions were methanol–water (5:95); the linear gradient started from this mobile phase to methanol–water (80:20) in 6 min. Isocratic elution was then held for 6 min and, finally, initial conditions were re-established in 1 min and held for 10 min. The flow-rate was 1 ml/min during all the gradient. Other chromatographic conditions were: room temperature; sample-loop, 20-μl; detection wavelength changing to improve the sensitivity of the determination: 283 nm for HMF and 337 nm for MA. Under these conditions, linear calibration graphs from 0·1 to 100 μg/ml for HMF and from 0·1 to 30 μg/ml for MA were obtained. Calibration graphs were prepared by plotting concentration against peak area.

Determination of HMF and MA in honey

Water extraction

A honey sample of 1 g was accurately weighed and dissolved in a 10-ml volumetric flask. The solution was homogenized, filtered through a 0·45 μm Millipore filter and injected into the chromatograph. The incomplete elution of the sample components will contribute significantly to the steady deterioration of the chromatographic column. For this reason, the column was washed with a 95:5 ethyl acetate–methanol mixture at the end of each day.

Methylene chloride extraction

A 5-g honey sample was weighed and extracted three times with 10-ml portions of methylene chloride. The organic extracts were mixed and evaporated at 30°C in a rotary evaporator to 2 ml. The solution was then injected into the chromatograph.

RESULTS AND DISCUSSION

A reversed-phase liquid chromatographic technique has been applied to the simultaneous determination of MA and HMF. Optimal mobile phase composition was determined in previous experiments with several binary solvent systems containing combinations of methanol with either water, acetonitrile, methylene chloride or ethyl acetate, as strength-adjusting solvents.

Figure 1 shows the dependence of isocratic capacity factors (K') on mobile phase composition for MA and HMF. The addition of organic modifiers, such as acetonitrile, methylene chloride or ethyl acetate, to methanol, caused MA and HMF to co-elute together in the void volume, even with very low proportions of the organic modifier in the mobile phase. Thus, a weaker solvent, water, was selected. With increasing water proportion, the K' values of MA increased markedly and those of HMF increased slightly.

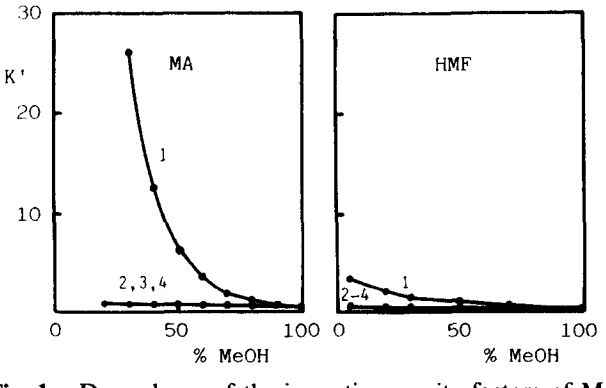

Fig. 1. Dependence of the isocratic capacity factors of MA and HMF on the composition of the mobile phase: 1, methanol–water; 2, methanol–acetonitrile; 3, methanol–ethyl acetate; 4, methanol–methylene chloride. Flow rate, 2 ml/min; UV detection, 283 nm for HMF, 337 nm for MA.

Table 1. Selectivity (α) and resolution (R_s) of MA and HMF

Mobile phase (2 ml/min)		α	R_s
MeOH:H_2O	96:4	1·42	0·80
	90:10	1·60	1·13
	80:20	1·76	2·07
	70:30	2·34	5·04
	50:50	5·74	12·76
	30:70	10·72	33·07
MeOH:ACN	80:20	1·23	0·35
	60:40	1·29	0·45
	40:60	1·26	0·65
MeOH:EtAc	90:10	1·34	0·67
	70:30	1·29	0·53
	50:50	1·21	0·40
MeOH:CH_2Cl_2	80:20	1·07	0·04
	60:40	0·97	0·04
	40:60	1·24	0·04
	20:80	0·91	0·08

To elucidate the optimal mobile phase for resolving the mixture with higher efficiency, values of separation factor (α) and resolution (R_s) were calculated and the results are shown in Table 1. For the methanol–water mixtures, both separation parameters increased at the higher water concentration; stronger solvents led to α values very close to 1 and R_s below 1, showing a poorer separation.

These results indicate that an adequate mobile phase for HMF implied a high proportion of water (above 70%) to ensure a good separation from the void volume avoiding possible interfering substances contained in the honey extracts which could be eluted at this volume. However, this mobile phase gave values of K' for MA near 20, thus leading to long analysis times and very broad peaks. Hence, a gradient elution technique was tried to obtain similar bandwidths for HMF and MA, faster overall separation and increasing detection sensitivity for MA.

The optimal solvent programme depends on the relationship of sample K' values (isocratic separation) to the composition of the mobile phase. The first step in designing the gradient entailed selection of the gradient profile. Since linear solvent strength gradients are satisfactory for most reversed-phase gradient-elution separations, a linear solvent programme was tried.

Table 2 illustrates the effect of varying the initial mobile phase composition, ϕ_0 (value of ϕ for mobile phase entering the column at time $t = 0$); the final value of ϕ at end of gradient, ϕ_f, the gradient steepness, ϕ' (rate of change of ϕ with time, expressed as % MeOH/min) and the time of gradient programme, t_s, on the instantaneous capacity factors of HMF and MA in gradient elution. Flow-rate was 1 ml/min in all experiments.

The effect of initiating the gradient with a mobile phase of higher strength (gradient 1 compared with 2) was that retention time of the earlier band (HMF) becomes shorter. The elution of the latter band (MA), however, was less sensitive to the starting composition of the solvent programme. Thus, an initial value of $\phi_0 = 5$ was selected to separate HMF from the void volume. The selected final fraction of methanol in the mobile phase was $\phi_f = 80$, since higher methanol concentrations would lead to long re-equilibration times of the column. Finally, optimal gradient steepness and total separation time were compared in gradient programmes 2, and 4 to 6. As t_s increased and ϕ' decreased, retention time of HMF was not significantly affected but MA was eluted later. Thus, a gradient time of 6 min (giving $\phi' = 12{\cdot}5\%$/min) was selected; this time minimized the separation time while it maintained adequate resolution and permitted HMF and MA to be successfully separated. This mobile phase was held for 6 min to ensure elution of all sample compounds of interest prior to the end of the gradient. After a gradient run, the column was regenerated to the initial mobile phase conditions, by running a 1 min reversed gradient followed by, at least, 10 min of isocratic operation at initial conditions. Table 3 shows the optimized gradient elution programme.

The chromatogram obtained for the HMF–MA mixture is shown in Fig. 2. Wavelength changes were required to improve the sensitivity of the determination.

Calibration graphs were obtained by plotting peak area against concentration and were linear in the overall range studied, 0·1–100 μg/ml (2–2000 ng) for HMF and 0·1–30 μg/ml (2–600 ng) for MA. The relative standard deviation (RSD) values for 2·62 μg/ml of HMF and 2·13 μg/ml of MA (10 determinations) were 2·3 and 3·8%, respectively.

Once the parameters necessary to run the reversed-phase gradient have been determined, a gradient of honey samples can be run. Several solvents were tried to prove that HMF and MA were quantitatively extracted from honey. Figure 3 shows the elution profiles

Table 2. Effect of mobile phase parameters on gradient elution capacity factors

Gradient	ϕ_0	ϕ_f	ϕ' (%/min)	t_s	K'_{HMF}	K'_{MA}
1	20	80	10·0	6	1·12	8·36
2	5	80	12·5	6	1·52	8·58
3	5	95	15·0	6	1·27	7·77
4	5	80	25·0	3	1·19	6·60
5	5	80	9·4	8	1·37	9·98
6	5	80	7·5	10	1·47	11·30

Table 3. Elution gradient programme (flow rate 1 ml/min)

Stage	Time (min)	Mode	Elution solvent (%)	
			MeOH	Water
Equil	10	Step	5	95
Start	6	Linear	80	20
End	6	Step	80	20
	1	Linear	5	95

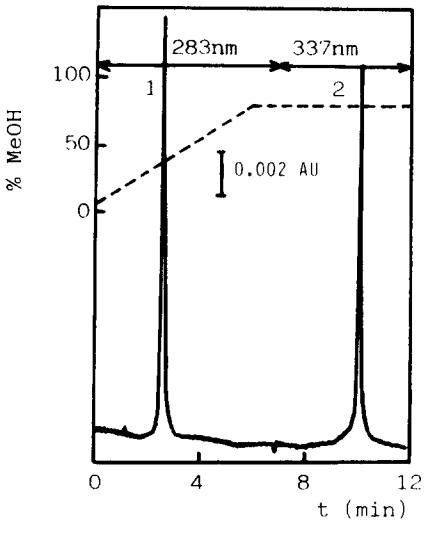

Fig. 2. Chromatogram of the HMF–MA mixture by reversed-phase gradient-elution. Flow-rate, 1 ml/min; sample-loop, 20 μl; profiles of solvent system and wavelength changes are reported at the top of the figure: (1) 1·45 μg/ml HMF; (2) 3·30 μg/ml MA.

corresponding to the injection of a honey sample dissolved in water (A) and a honey sample extracted three times with methylene chloride (B). As can be seen, in the water solution (chromatogram A) only the peak corresponding to HMF can be detected. This extraction procedure was not sufficiently sensitive to determine MA. However, in the extract of methylene chloride (chromatogram B) two peaks can be located at the retention times corresponding to HMF and MA. This is because three extracts have been obtained from the honey sample and the resulting solutions were mixed and evaporated to 2 ml; thus, this solution must be 25-fold more concentrated in MA than the aqueous one, allowing the quantification of both HMF and MA.

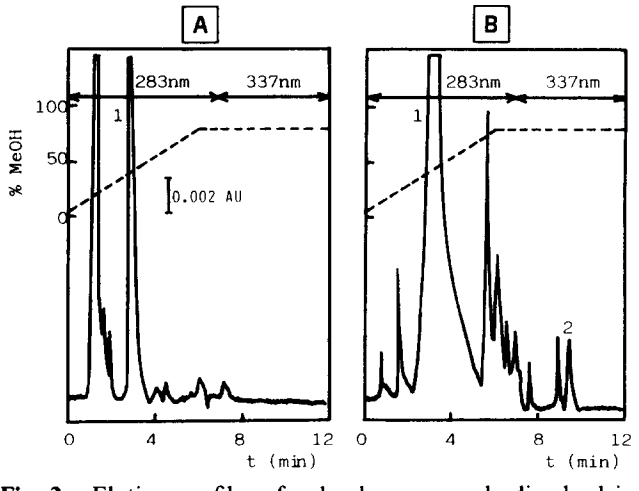

Fig. 3. Elution profiles of a 1-g honey sample dissolved in water (A) and a 5-g honey sample extracted with methylene chloride (B). Flow-rate, 1 ml/min; sample-loop, 20-μl; profiles of solvent system and wavelength changes are reported at the top of the figure: (1) HMF; (2) MA.

A comparison of both solvents, water and methylene chloride, as extractants, might conclude that if only HMF, as a quality factor of the honey, must be determined, water should be the preferred solvent because of the simplicity of the extraction procedure. However, if both HMF and MA contents must be determined, a methylene chloride extraction must be recommended due to the very low content of MA.

In the authors' opinion, the peak shape for HMF is due to the high concentration of this compound in the methylene chloride extract. Since MA is present in very low concentrations (below 1 μg/g) in the honey, a very concentrated honey extract must be prepared to obtain an accurate quantification of MA. On the other hand, HMF is highly concentrated in this extract and, for this reason, the peak appears with a poor shape. It was proved that the shape of the peak does not affect the quantification of HMF. This problem is not observed in the water extract because, as indicated above, the water solution must be 25-fold more diluted in HMF than the methylene chloride extract. If a more diluted organic extract of honey is prepared, the peak of HMF is much more defined.

In the range studied, 0·5–2·0 ml/min, the mobile phase flow-rate was not a significant factor affecting the separation. A 1 ml/min value, which gave a slightly better resolution between MA and other peaks from the extract of honey, was selected.

Several honey samples from different Spanish geographic areas were analysed and results for the contents of HMF and MA are presented in Table 4. Three aliquots were taken for each sample and each one of the extracts was three times injected into the chromatograph. The samples were also analysed by adding

Table 4. HMF and MA contents in Spanish honeys

Sample	HMF (μg/g)		MA (μg/g)	
	Water ext.	CH_2Cl_2 ext.	Water ext.	CH_2Cl_2 ext.
1	22·7	19·4	ND	0·73
2	41·5	37·0	ND	0·23
3	54·8	48·6	ND	0·22
4	49·5	41·4	ND	0·56
5	47·5	40·5	ND	0·18
6	39·5	39·2	ND	ND
7	146·0	132·5	ND	ND

Table 5. Recovery of HMF and MA from honey by the standard addition method

HMF present (μg/ml)	HMF added (μg/ml)	Recovery (%)	MA present (μg/ml)	MA added (μg/ml)	Recovery (%)
55·6	16·9	102·6	0·62	2·76	98·4
55·6	33·8	103·5	0·62	5·52	97·2
38·9	16·9	98·7	0·43	2·76	96·3
38·9	33·8	102·9	0·43	5·52	95·0

37·5 μg/ml of HMF and 3·0 μg/ml of MA standards to the solution. Results agree with those obtained for non-spiked samples. From the results shown in Table 4, it is evident that the HMF contents obtained from the aqueous solutions were slightly higher than those obtained when the organic extracts were analysed. These discrepancies were attributed to analyte losses in the whole analysis process. In the authors' opinion, the simplicity and minimal manipulation of the sample gives more analytical reliability to the water-based procedure. There is no doubt that sample 7, which was purchased in a supermarket, shows a very high HMF content, proving that the product was submitted to inappropriate processing or storage conditions or is suspect of adulteration.

A previous study (White, 1965) of the MA content of *Citrus* honey reported an average value of 2·87 μg/g. However, a considerable variation among samples from different areas and seasons was noted. On the other hand, it has been suggested (Serra, 1988) that samples containing more than 0·5 μg/g can be classified as *Citrus* honeys. Samples 1–4 were obtained from geographic areas having extensive plantations of *Citrus*. Although these samples showed higher concentrations in MA than samples 5–7, no conclusions on their classification as *Citrus* honeys can be obtained due to lack of data on their collection and storage conditions before the analysis.

The recoveries of HMF and MA were determined by the analysis of methylene chloride honey extracts spiked at levels from 16·9 to 33·8 μg/ml of HMF and from 2·8 to 5·5 μg/ml of MA; results are shown in Table 5.

Several experiments were carried out to study the reproducibility of the method. Ten injections of the same honey extract were used to determine the instrument stability (relative standard deviation for HMF: 4·0%). To calculate the precision of the whole procedure, 10 separate extractions were performed from the same sample and the solutions were chromatographed. The relative standard deviation was now 4·9%.

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