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Journal of Chromatography A, 665 (1994) 105–109

JOURNAL OF CHROMATOGRAPHY A

Short Communication

# Application of high-performance liquid chromatography to the analysis of the complex volatile mixture of blackcurrant buds (*Ribes nigrum* L.)

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**Abstract**

The essential oil of blackcurrant buds was fractionated into hydrocarbons and oxygenated compounds and the two fractions were submitted to reversed-phase high-performance liquid chromatographic (HPLC) column. Volatile carbonyls belong among the most important compounds for the blackcurrant flavour and were therefore analysed in detail. The carbonyls were converted into 2,4-dinitrophenylhydrazones and the mixture of 2,4-dinitrophenylhydrazones was separated into derivatives of keto acids and monocarbonyl and dicarbonyl compounds. Each fraction was submitted to HPLC. The results of the HPLC separations are discussed.

## 1. Introduction

The most important part of the blackcurrant shrub for flavour isolation is the dormant buds. The essential oil of blackcurrant buds gives off a strong terpenic flavour overwhelmed by a catty note [1]. Although the hydrocarbon fraction represents the major part of the oil, it does not explain the blackcurrant odour. The oxygenated compounds represent the most odorous volatile components and exhibit the characteristic blackcurrant odour [2,3]. The volatile compounds in blackcurrant buds have been investigated [3–6] using gas chromatography, mass spectrometry and infrared spectrometry. The application of high-performance liquid chromatography (HPLC) in aroma research is still in a stage of development. In the literature emphasis is put on the potential of this method for the separation of aroma concentrates. An effective HPLC method has been used for prefractionation of monoterpene and sesquiterpene hydrocarbons from the oxygenated compounds [4,7]. Liquid chromatography can be applied to subdivide an aroma concentrate into fractions in order to judge them sensorially.

This study was undertaken in order to increase the knowledge of essential oil analysis and blackcurrant bud volatiles. The objectives were to separate the volatile mixture into hydrocarbons and oxygenated compounds and to precipitate the volatile components with a carbonyl group.

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0021-9673/94/$07.00 
*SSDI* 0021-9673(93)E0037-U

The results of HPLC separations of hydrocarbons, oxygenated compounds and carbonyls are described. The derivatives of carbonyl compounds have been separated previously only using thin-layer chromatography [8].

## 2. Experimental

Dormant buds (*Ribes nigrum* L., variety Eva) were harvested from cuttings during February 1993. The variety is descended form a crossing of Silvergieter's Zwarte × Holland Black. The buds were stored at −21°C before use. All reagents used (HPLC grade) were obtained from Lachema (Brno, Czech Republic), except for methanol and pentane (HPLC grade), which were obtained form Merck (Darmstadt, Germany).

### 2.1. Analysis of blackcurrant bud essential oil

Dormant buds (100 g) were mixed with 500 ml of distilled water and comminuted in a blender for 3 min. The volatile components were steam distilled in a Likens–Nickerson extractor (constructed by Mr. Greif of this department) for 45 min with pentane (100 ml) as solvent. The pentane extract was dried over anhydrous $Na_2SO_4$. The solution was filtered and the pentane was evaporated on a water-bath at 40°C under atmospheric pressure through a Vigreux column (Kavalier, Sázava and Sáz, Czech Republic) to 0.5 ml. The concentrate was fractionated in a jacketed column at 11°C on a 5 g of silica gel (0.2–0.5 mm) (Merck) hydrated to 15% (w/w). The hydrocarbons were eluted with 50 ml of pentane and the oxygenated fraction was eluted with 40 ml of diethyl ether. The eluates were dried over anhydrous $Na_2SO_4$ and concentrated to 0.4 ml for HPLC analysis. A HPP 4001 high-pressure pump equipped with a LCD 2563 UV–Vis detector and a TZ 4620 line recorder (Laboratorní přístroje, Prague, Czech Republic) were used. Separations were performed using a Separon SGX $C_{18}$ reversed-phase column (150 mm × 3 mm I.D.), particle diameter 5 μm (Tessek, Prague, Czech Republic). The operating conditions were as follows: mobile phase, methanol–water [4:1 (v/v) for the hydrocarbon fraction and 1:1 (v/v) for the oxygenated fraction], sensitivity, 8; UV detection at 254 nm; injection volume, 0.5 μl (LCI-30 injector; Laboratornmí přístroje).

### 2.2. Analysis of the carbonyls of blackcurrant bud volatiles

Dormant buds (250 g) were mixed with 1000 ml of distilled water and comminuted in a blender for 4 min. The volatile components were steam distilled in a distillation apparatus at atmospheric pressure. The ultimate volume of a distillation product was 200 ml.

Approximately 25 ml of a 1% solution of 2,4-dinitrophenylhydrazine in 7.5% hydrochloric acid were added to the distillation product. The suspension was heated at 100°C for 5 min and then cooled to room temperature. After standing overnight, the suspension with precipitated 2,4-dinitrophenylhydrazones (2,4-DNPHs) was filtered and the excess of reagent removed by washing with 7.5% hydrochloric acid until the effluent becomes colourless. The residual HCl was then removed by washing with water. Precipitated 2,4-DNPHs were dissolved in 100 ml of chloroform.

The chloroform solution of 2,4-DNPHs was extracted by shaking five times with 10 ml of 10% sodium carbonate solution to remove the 2,4-DNPHs of keto acids. After adjustment of the pH to 3 with 85% phosphoric acid, the 2,4-DNPHs of keto acids were extracted by shaking five times with 10 ml of chloroform. The chloroform extract was dried over anhydrous $Na_2SO_4$ and concentrated on a water-bath of 0.5 ml.

The chloroform solution of 2,4-DNPHs was dried over anhydrous $Na_2SO_4$ and heated at 62°C to remove the solvent. The dry residue was extracted by shaking five times with 10 ml of *n*-hexane to dissolve the mono-2,4-DNPHs. The hexane extract was concentrated on a water-bath to 0.5 ml.

The dry residue was dissolved in 50 ml of chloroform. The chloroform solution of bis-2,4-

DNPHs was concentrated on a water-bath to 0.5 ml.

Each fraction was submitted to HPLC analysis. The conditions were the same as for the analysis of the blackcurrant bud essential oil except that the mobile phase was methanol–water (4:1, v/v) for each fraction and UV detection was carried out at 365 nm.

## 3. Results and discussion

Steam distillation–extraction of the buds allows the recovery of 0.4–0.6 ml of essential oil per 100 g of buds.

In the literature emphasis is put on the potential of HPLC for the separation of aroma concentrates. However, HPLC analysis of a total essential oil usually does not give a complete separation of all of the components present, the peaks of the hydrocarbon constituents often overlapping with those of oxygen-containing compounds. Prefractionation of a naturally occurring mixture of essential oils by column chromatography is effective in separating hydrocarbons from oxygenated compounds and leads to a better HPLC separation. For this reason the blackcurrant bud essential oil mixture was fractionated into hydrocarbons and oxygenated compounds by column chromatography on silica gel by elution with pentane and diethyl ether.

The HPLC traces for the hydrocarbons and oxygenated compounds are shown in Figs. 1 and 2. In a reversed-phase HPLC column, increasing numbers of carbon atoms cause an increase in retention time, but an increase in the number of carbon–carbon double bonds cause a decrease in retention time [9]. As a consequence of using a reversed-phase HPLC column, the sesquiterpenes and sesquiterpenoids have longer retention times than monoterpenes and monoterpenoids and this provides a basis for peak identification. Injection of pure $\alpha$-pinene, $\beta$-pinene, $\alpha$-terpinene, 3-carene, *m*-cymene, *p*-cymene (Aldrich, Steinheim, Germany), $\beta$-caryophyllene and $\alpha$-humulene (Sigma, St. Louis, MO, USA) resulted in a chromatographic profile of monoterpenes in the time period from

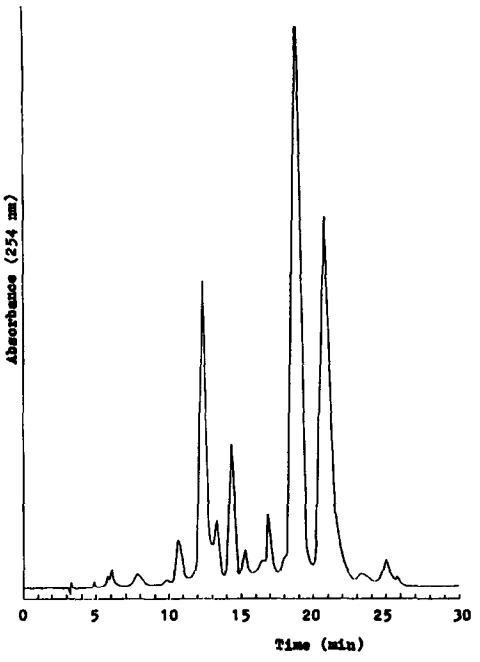


Fig. 1. HPLC separation of volatile hydrocarbons of blackcurrant buds. Reversed-phase HPLC column, 150 mm × 3 mm I.D., Separon SGX $C_{18}$; mobile phase, methanol–water (4:1, v/v); flow-rate, 0.3 ml/min; UV detection at 254 nm.

4 to 18 mins and that of sequiterpenes from 18 to 26 mins (Fig. 1).

The most important compounds for the aroma of blackcurrant bud oil are present in the polar fraction. These polar volatiles contain the most odorous compounds and exhibit the characteristic blackcurrant odour. Volatile carbonyls, present in a very small amounts, belong among the most important compounds for the blackcurrant flavour. For this reason, the carbonyls were analysed in detail. The carbonyl compounds were converted into 2,4-DNPHs. The mixture of 2,4-DNPHs obtained from blackcurrant bud volatiles is a complicated mixture consisting of the derivatives of saturated and unsaturated aliphatic aldehydes and ketones, terpenoids and aromatic-type carbonyl compounds. Before analysis by HPLC it is advisable to separate the mixture into classes containing one type of carbonyl compound. The mixture of 2,4-DNPHs was separated into derivatives of keto acids and monocarbonyl and dicarbonyl compounds. Each

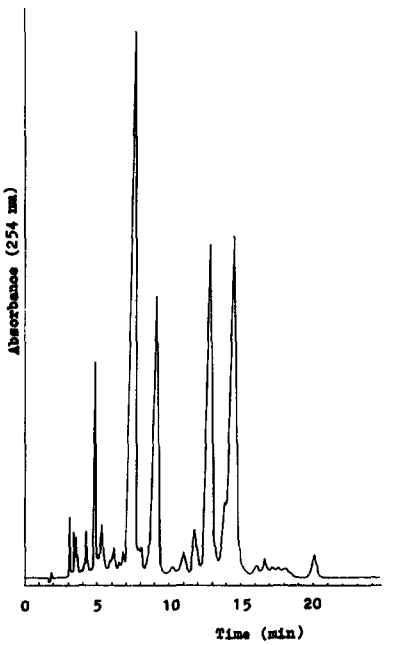


Fig. 2. HPLC separation of volatile oxygenated compounds of blackcurrant buds. Conditions as in Fig. 1 except mobile phase, methanol–water (1:1, v/v).

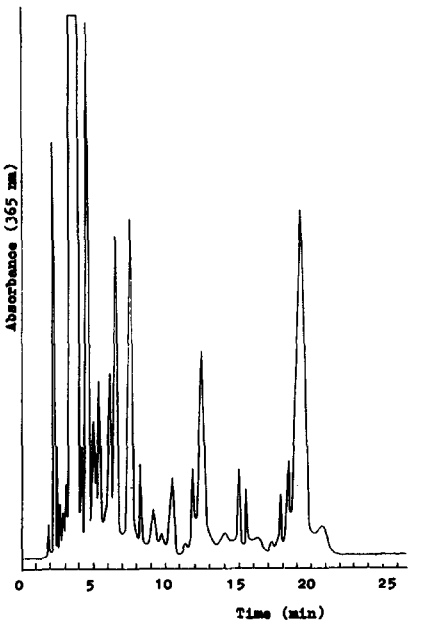


Fig. 4. HPLC separation of 2,4-DNPHs of monocarbonyl compounds. Conditions as in Fig. 1 except UV detection at 365 nm.

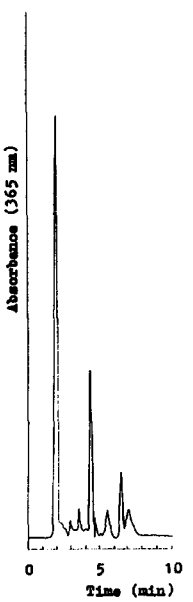


Fig. 3. HPLC separation of 2,4-DNPHs of keto acids. Conditions as in Fig. 1 except UV detection at 365 nm.

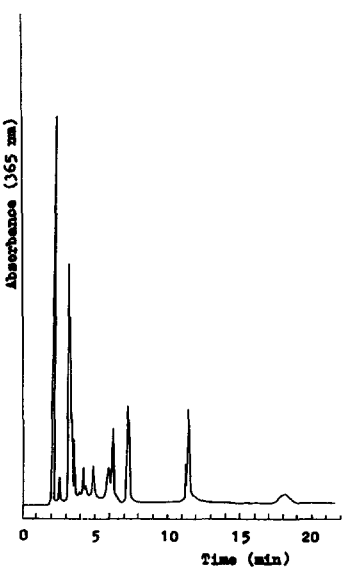


Fig. 5. HPLC separation of 2,4-DNPHs of dicarbonyl compounds. Conditions as in Fig. 1 except UV detection at 365 nm.

fraction was submitted to HPLC (Figs. 3, 4 and 5). As can be seen, monocarbonyl compounds represent the major part of the carbonyl volatiles. It will be necessary to use, after regeneration, gas chromatography–mass spectrometry for identification of the carbonyl compounds.

## 4. Conclusions

Reversed-phase HPLC appears to be useful for the analysis of essential oils and 2,4-DNPHs. As is shown in Figs. 1–5, HPLC was suitable for testing the essential oils and the fingerprint method. The low temperatures at which separation takes place is an advantage over gas chromatography, and is especially important when dealing with thermolabile compounds such as many occur in essential oils.

## 5. References

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