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Journal of Chromatography A, 806 (1998) 349–354

JOURNAL OF CHROMATOGRAPHY A

Short communication

Quantitative determination of trace and ultratrace flavour active compounds in red wines through gas chromatographic–ion trap mass spectrometric analysis of microextracts

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Received 2 October 1997; received in revised form 23 January 1998; accepted 23 January 1998

Abstract

A GC–MS method for analysing the most important flavour active odorants of wine has been developed. The method combines a two-step preconcentration stage (demixing+microextraction) and a GC–ion trap MS determination. In the method, 50 ml of wine are previously adjusted to 13% (v/v) alcohol, and the alcoholic fraction is demixed by salting out. That fraction is partially rediluted, extracted with 0.1 ml of 1,1,2-trichlorotrifluoroethane (freon 113) and analysed by GC–MS to obtain quantitative information on 25 analytes whose concentrations range from 0.1 to more than 1000 μg/l. Those analytes are esters, alcohols, terpenols, aromatic ketones, lactones, ethers and volatile phenols. The overall method R.S.D. ranges from 3 to 7%, and the linear behaviour is very good except for the most concentrated analytes. Standard addition experiments and analyses of spiked samples have demonstrated that both the MS quantification and the overall method are free of matrix effects, and that only two internal standards are needed. The limits of detection range from 20 to 1000 ng/l, and all the analytes can be detected at the concentration in which they become flavour active. © 1998 Elsevier Science B.V.

Keywords: Wine; Food analysis; Aroma compounds; Sample preparation

1. Introduction

There are nearly 40 flavour active molecules in the volatile fraction of a red wine, which represents only about a 5% out of the total of this fraction [1]. These odorants belong to quite different chemical classes: alcohols, esters, volatile phenols, terpenols, lactones, aromatic ketones and ethers; their concentrations range from less than 1 μg/l (aromatic ketones) to more than 100 mg/l (some alcohols) [2,3]; and they have quite different chemical and physicochemical properties. Thus, the isolation step must be rather non-selective and should provide a strong preconcentration; and a very high selectivity is required in the further chromatographic separation and quantification steps. There are two main strategies to achieve the desired selectivity: to combine several chromatographic separation steps, which was explored in a recent paper [4]; or to use mass spectrometry (MS) which is the main subject of this paper. In the literature, only a couple of methods based on gas chromatography (GC)–MS have been proposed, [5,6], but these are only designed to analyse the wine content in terpenic compounds.

With regard to the isolation and preconcentration steps, the most widely used methods include purge

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0021-9673/98/$19.00
PII S0021-9673(98)00070-3

and trap [7], liquid–liquid extraction methods [8–10], lipophylic resin extraction [11], supercritical fluid extraction [12] or demixing/microextraction [4,13]. From the point of view of extract cleanliness, the preferred sample preparation methods would be purge-and-trap-based methods, but they do not provide the necessary sensitivity for the analysis of the medium- to high-boiling flavorants. The extracts based on a direct continuous liquid–liquid extraction should not be used for routine analyses, unless an additional cleaning step is included. On the other hand, lipophylic resin extraction cannot be directly accomplished on the wine, but on its distillate, which complicates the analysis and can bring about the formation of artifacts. In contrast, the demixing/microextraction approach may be very appropriate for GC–MS analysis, since: (i) it avoids using the imprecise solvent evaporation steps [14]; (ii) extraction is performed at laboratory temperature; (iii) ultra high purity solvents are not required, and (iv) the final extract can be clean enough if the extraction conditions are correctly chosen. This is the approach that will be explored in the present paper to develop an analytical method able to quantify the most important odorants found in wine.

2. Experimental

2.1. Materials and equipment

2.1.1. Solvents

Absolute ethanol ARG was from Panreac (Barcelona, Spain); freon 113 (1,1,2-triclorotrifluoroethane) HPLC grade from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.1.2. Chemical standards

Analytical grade standards were from Fluka (Buchs, Switzerland), Sigma (St. Louis, MO, USA), Aldrich (Steinheim, Germany), Lancaster (Morecambe, UK), Chemservice (West Chester, PA, USA) and Polyscience (Niles, IL, USA).

2.1.3. Standard and auxiliary solutions

2.1.3.1. Individual calibration solutions

Exact masses ($>0.0300\pm0.0001$ g) of the chemical standard compounds were dissolved in absolute ethanol and made up to volume (10 ml).

2.1.3.2. Mixed ethanolic (or freonic) standard solution

Chemical standards were dissolved in ethanol (or freon 113) at concentrations 2 or 3 orders of magnitude higher than those typically found in wine. These solutions were then diluted with water and/or alcohol (adjusting final alcohol content to 13%, v/v) to prepare the calibration graph(s) and to spike different wine samples. All the synthetic wine samples used in the calibration graph were 5 g/l of tartaric acid and pH 3.4 adjusted with 1 *M* NaOH.

2.1.3.3. Internal standard solution

4-Methyl-2-pentanol, 2-octanol and *n*-dodecanol, 0.1 mg/g in ethanol.

2.1.3.4. Saline solution

34.85 g $(NH_4)_2SO_4$ dissolved in 100 ml water.

2.1.4. Gas chromatography–mass spectrometry conditions

A Star 3400CX gas chromatograph fitted to a Saturn 4 electronic impact mass spectrometer from Varian was used. The analytical column was DB-WAX (J&W Scientific, Folsom, CA, USA) 60 m$\times$ 0.32 mm; 0.5 μm film thickness, preceded by a 2 m$\times$0.32 mm retention gap. The carrier gas was helium at 1 ml/min. The temperature program was: 40°C for 5 min, then raised to 190°C at 2°C/min. Transfer line temperature was 220°C. Injection was by an A 1093 SPI (septum-equipped programmable injector) from Varian: initial temperature 30°C for 6 s, and then raised to 190°C at 200°C/min; the injection volume was 1 μl.

Mass spectrometry: mass range, m/z 35–200 (except the last 2.67 min when it was m/z 99–99), 1 scan/s. The filament current was held at 19 μA. Some segments of the chromatogram were registered in MS–MS under the conditions given in Table 1.

Table 1
MS–MS conditions for the analytical determination of some compounds

Analyte	Parent ion mass (m/z)	Excitation time (ms)	Excitation amplitude (V)
Furfural	95.0	20	0.00
α-Ionone	121.0	20	45.00
Guaiacol	124.0	20	45.00
β-Ionone	177.0	20	40.00
Ethyl cinnamate	131.2	20	45.00
γ-Decalactone	85.0	20	0.00
Eugenol	164.0	20	40.00
4-Vinylguaiacol	150	20	40.00

2.2. Proposed method

2.2.1. Preconcentration by demixing (as explained in Ref. [4]*)*

Add 0.075 g (ca. 100 ml) internal standard solution to a 50-ml volumetric flask previously wetted with the wine to be analysed, immediately after this, add more wine to ensure that the internal standard solution does not evaporate, shake to mix, and finally make up to volume with the wine sample. Add the necessary amount of ethanol to adjust the ethanol content to 13% (v/v). To a dry separating funnel (250 ml), add 6.57 g $NaH_2PO_4 \cdot H_2O$, 27 g $(NH_4)_2SO_4$ and, finally, the 50 ml of wine normalised to 13% (v/v) ethanol. Shake until salt is completely solved (ca. 10 min), and let the phases separate for at least 3 h.

2.2.2. Freon 113 microextraction

Pipette an aliquot of the supernatant organic phase (2 ml) into a screw capped centrifuge tube (15 ml), add saline solution (5 ml) and freon 113 (0.1 ml). Cap the tube and leave it shaking fast for 1 h. After this, centrifuge the tube at 3000 rpm for 5 min. Recover and analyse the freon extract.

2.2.3. Measurement of analyte concentration

Inject the organic phase in the chromatographic system under the conditions given above using the quantitative conditions described in Table 2 and interpolate the relative response (area or height) versus the corresponding internal standard in the calibration graph prepared by analysing synthetic wines containing different amounts of the volatile components. The range of concentrations considered in this study can be seen in Table 3.

2.3. Method optimisation and validation

2.3.1. MS and MS–MS quantitative fragments

Mass selective fragments were chosen through the injection of freon 113 standard samples and of freon 113 extracts of wine and spiked wine samples. The repeatability and linear performance of injection and detection were also studied through a standard addition experiment performed on wine freon 113 extracts.

2.3.2. Method reproducibility and determination of the existence of matrix effects

Three different red wines (young grenache, 2-year-old cabernet and 5-year-old merlot) were used in this experiment. Each wine was spiked with a known amount of mixed standard ethanolic solution (see Table 4), and both the wines and the spiked samples were analysed in triplicate following the proposed procedure.

3. Results and discussion

The compounds that constitute the main aim of this research are listed in Table 2 together with the MS conditions used in their quantification. It can be seen that the major compounds in the chromatogram were best quantified by measuring their total reconstructed peak area, while the minor compounds required selected fragments, or even MS–MS conditions (given in Table 1). The results of the study of the analytical performance of the chromatographic–spectrometric process are shown in Table 2. Reproducibility of the GC–MS operation is satisfactory in most cases: it is well below 3% in 22 cases, and over 5% only in two cases. Linearity behaves in quite a different way depending on the concentration level of the compounds. It is, in general, very good for the minor compounds, and not so good for the major ones (data not given). It should be noted, however, that the very good reproducibility figures found show that a calibration is possible in that cases, although not a linear one. The slopes found in the standard addition experiment were compared with the ones

Table 2
Quantitative conditions used in this study

Analyte	Quantitative m/z	I.S.[a]	R.S.D. (%)[b]	Range (mg/l)[c]	D.L. (μg/l)
Ethyl isobutyrate	35–200	4M2P	4.21	6.6–43.5	30
Ethyl butyrate	71, 88, 116	4M2P	1.75	14.7–395	18
Ethyl 2-methylbutyrate	84, 102, 115	4M2P	8.06	0.5–4.4	20
Ethyl isovalerate	57, 103, 131	4M2P	1.64	0.7–4.1	74
Isoamyl acetate	35–200	4M2P	2.00	224–601	113
Ethyl hexanoate	35–200	4M2P	1.19	49.4–415	27
cis-3-Hexenol	35–200	4M2P	1.01	13.5–408	37
Ethyl octanoate	35–200	4M2P	1.18	55.7–426	52
Furfural	95, 96	4M2P	4.11	0.1–6.9	8
Linalool	93, 121, 136	2-Octanol	1.45	3–34.2	17
Ethyl furoate	95	4M2P	4.98	0.1–38.2	2.5
Ethyl decanoate	35–200	4M2P	2.52	14.0–52.4	23
Ethyl benzoate	105	4M2P	1.86	0.1–3.7	2.5
α-Terpineol	93, 121, 136	4M2P	1.48	0.9–35.8	5.5
Phenylethyl acetate	35-200	4M2P	2.10	23.0–388	133
Geraniol	69, 123, 139	2-Octanol	0.81	2.3–37.0	24
α-Ionone	77, 91, 121	4M2P	3.30	0.1–4.2	12
Guaiacol	81, 109, 124	4M2P	2.54	0.4–4.3	21
β-Ionone	147, 161, 177	4M2P	1.68	0.1–4.2	6
γ-Nonalactone	85, 157	2-Octanol	1.65	3.1–36.9	22
Ethyl cinnamate	103, 131	2-Octanol	1.32	0.2–4.3	17
γ-Decalactone	57, 85	4M2P	6.41	0.3–3.7	66
Eugenol	104, 121, 131	4M2P	2.69	0.1–5.1	18
4-Vinylguaiacol	107, 135, 150	4M2P	2.58	0.2–4.5	64
δ-Decalactone	99	2-Octanol	3.20	0.3–3.7	53

Analytical characteristics of the GC–MS analysis.
[a] Internal standard. 4M2P: 4-methyl-2-pentanol.
[b] Data based on six standard deviations with two degrees of freedom each.
[c] Concentrations referred to the freon 113 extract.

found in synthetic solutions in order to check the existence of matrix effects. The results (data not shown) were very satisfactory, and the only cases in which real significant differences were found were those in which the linear approximation was not correct (isoamyl acetate and ethyl decanoate). Detection limits given in the table refer to absolute concentration in the extract (not in the wine). The lowest figure found is 2.5 μg/l, and the average value is around 20 μg/l. It should be remarked that these values are one order of magnitude lower than those found in a standard operation with flame ionisation detection, and almost two orders of magnitude lower than those found with standard scan monitoring detection in bench top quadrupoles.

Once it has been concluded that it is feasible to use an ion trap, there remains the question of the sample preparation scheme that provides an optimal solution. A good compromise is the use of the demixing/microextraction steps of the method previously developed [4], because of its reproducibility, accuracy and simplicity. It was found that the microextraction method had to be readjusted to get cleaner and more concentrated extracts, which was achieved by adjusting the alcoholic content of the aqueous phase, the volume of solvent and the amount of salt added. The final protocol provides a 0.1 ml microextract clean enough to be injected directly into the GC–MS system.

A set of recovery experiments, including now the sample preparation steps, was carried out to look for the existence of matrix effects and to estimate the analytical characteristics of the complete analytical method. The results on method reproducibility are given in Table 3. Data have been split to show averaged variances at low (the samples) or high level

Table 3
Analytical characteristics of the global method: method reproducibility, linearity and detection Limits

Compound	%R.S.D. low level	%R.S.D. high level	Slope	r^2	n	Linear range (μg/l)	D.L. (μg/l)
Ethyl isobutyrate	5.33	3.49	2.50	0.9992	5	9.6–191	0.33
Ethyl butyrate	3.43	3.78	0.606	0.9982	5	46–926	0.08
Ethyl 2-methylbutyrate	8.54	4.93	0.486	0.9984	5	0.4–9.0	0.12
Ethyl isovalerate	4.88	5.96	0.366	0.9999	6	0.9–44	0.15
Isoamyl acetate	3.87	4.32	2.07	0.9959	5	500–9300	0.38
Ethyl hexanoate	4.49	4.90	3.42	0.9991	5	90–1900	0.41
cis-3-Hexenol	5.56	1.96	0.393	0.9996	5	9.6–193	4.15
Ethyl octanoate	4.31	6.81	3.95	0.9974	5	193–3859	0.14
Furfural	11.2	13.6	0.027	0.9977	4	0.9–8.9	0.67
Linalool	2.27	3.15	0.167	0.9999	5	2.0–39.8	0.15
Ethyl furoate	5.70	3.15	0.418	0.9993	6	0.2–8.8	0.12
Ethyl decanoate	5.27	6.81	3.53	0.9919	5	31.5–631	0.38
Ethyl benzoate	3.95	4.63	1.40	1.0000	6	0.2–9.2	0.07
α-Terpineol	5.51	4.44	0.694	0.9959	5	2.9–58	0.11
Phenylethyl acetate	3.58	3.65	3.57	0.9960	4	45.2–452	0.18
Geraniol	5.09	4.71	0.169	0.9985	5	2.1–42	0.15
α-Ionone	9.63	4.10	0.529	0.9995	6	0.09–4.7	0.05
Guaiacol	9.52	5.86	0.014	0.9959	3	1.8–8.9	1.06
β-Ionone	5.38	7.23	0.075	0.9981	6	0.09–4.6	0.02
γ-Nonalactone	5.81	4.89	0.182	0.9993	5	2.0–40.6	0.38
Ethyl cinnamate	6.56	3.37	0.110	0.9944	6	0.09–4.7	0.04
γ-Decalactone	8.86	4.85	0.124	0.9954	3	0.9–4.4	0.44
Eugenol	6.56	6.71	0.056	0.9999	3	0.9–4.6	0.36
4-Vinylguaiacol	14.25	9.53	0.016	0.9989	3	1.8–9.0	0.93
δ-Decalactone	7.19	4.73	0.0189	0.9991	3	0.5–4.7	0.47

n=Number of points in the calibration graph.

of concentration (the spiked samples). The data in the Table show that the averaged values range from 3 to 7% for most of the compounds, and that they are only worse than 10% for furfural and 4-vinylguaicol, which can be considered quite satisfactory. The limits of detection can also be seen in Table 3. The lowest value is that of β-ionone (20 ng/l), while the worst values are those of *cis*-3-hexenol, guaiacol and 4-vinylguaiacol. For most of the studied analytes, the detection limits are well below 0.5 μg/l. These values can be considered satisfactory, since all the quantification limits are well below the sensory thresholds of these compounds [2,3]. Results of linearity are given in Table 3, where it can be seen that the behaviour is quite satisfactory, except in the aforementioned cases of isoamyl acetate, and ethyl decanoate.

Table 4 gives the results obtained in the analysis of three spiked samples. The three samples were different but were spiked with the same amounts of analytes. A one-way analysis of variance (ANOVA) experiment was carried out with the results in order to check the existence of matrix effects. An asterisk marks the only case that was found to differ, and it was β-ionone. The concordance between the added and recovered amounts of analyte was fairly good in all cases.

In conclusion, all this research has allowed us to develop a reliable and powerful method, able to quantify the most important flavour-active compounds of a wine.

Acknowledgements

This work has been funded by the Spanish CICYT (Comisión Interministerial de Ciencia y Tecnología), project ALI 95-0475 and by the Regional Government of Aragón, project CONSID P20/96.

Table 4
Analytical characteristics of the global method: sample composition and analyte recoveries in three different samples

Compound	Added (μg/l)	Sample 1 (μg/l)	Sample 2 (μg/l)	Sample 3 (μg/l)	Recovery (%)
Ethyl isobutyrate	15.2	27.8	62.9	28.6	95.2±9
Ethyl butyrate	62.3	292	189	199	95.7±5
Ethyl 2-methylbutyrate	12.8	1.92	10.9	18.3	103±2
Ethyl isovalerate	25.0	5.30	15.0	24.8	97.1±6
Isoamyl acetate	409	1333	370	251	99.0±4
Ethyl hexanoate	73.3	440	282	260	96.4±10
cis-3-Hexenol	66.2	99.8	303	323	95.7±9
Ethyl octanoate	161	542	307	235	96.9±10
Furfural	2.0	4.26	46.1	98.3	81.7±25
Linalool	10.7	10.9	14.0	58.1	101±8
Ethyl furoate	4.9	3.95	8.57	19.2	107±14
Ethyl decanoate	87.0	179	87.9	42.4	91±11
Ethyl benzoate	5.4	0.48	0.80	1.99	107±6
α-Terpineol	15.1	3.31	15.2	44.3	106±9
Phenylethyl acetate	43.0	59.2	3.24	4.98	91±8
Geraniol	17.7	10.1	6.16	22.9	102±2
α-Ionone	2.5	<0.09	0.23	0.81	104±7
Guaiacol	3.8	0.99	5.59	12.1	121±29
β-Ionone	0.5***	0.72	0.89	1.02	173±162
γ-Nonalactone	14.1	26.0	18.7	24.1	98±13
Ethyl cinnamate	4.5	1.05	0.99	0.47	98±2
γ-Decalactone	1.9	1.19	nd	2.16	105±10
Eugenol	2.4	1.52	3.01	14.1	97±2
4-Vinylguaiacol	3.1	4.04	0.44	9.20	94±6
δ-Decalactone	1.7	4.97	6.28	1.23	99±7

nd=Not detected.
*** Differences significant at $p>0.05$.

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