Determination of indole-3-acetic acid, tryptophan and other indoles in must and wine by high-performance liquid chromatography with fluorescence detection

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Abstract

The development of a robust method to analyse the content of tryptophan and of indole-3-acetic acid at the microgram per litre level in must and wine is necessary in order to study the formation of 2-aminoacetophenone and of other indole compounds causing the 'untypical ageing off-flavour'. The present paper discusses the development and validation of a reversed-phase high-performance liquid chromatography method with fluorescence detection for the analysis of indole-3-acetic acid, tryptophan, tryptophol, indole and skatole in must and white wine. The required selectivity and sensitivity was gained through the solid-phase extraction on a polystyrene-based polymer column. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

In recent years, an apparent alteration in the flavour of white wines, named 'untypical ageing off-flavour' has been frequently reported in white wines from Central Europe [1–3]. This undesired organoleptic character has been demonstrated to be closely related to the appearance in wine of 2-aminoacetophenone (2-AAP) [1], which can spoil the aroma of wine at a level close or slightly over one microgram per litre [4–6]. The detailed mechanism of formation of 2-AAP has not been ascertained to date. It has been clearly demonstrated that the most likely pathway of formation of 2-AAP is from the degradation of L-tryptophan (TR) and/or indole-3-acetic acid (IAA), which are present in must, during the winemaking process [6–11]. As a result, one or more unstable — and up to now unknown — intermediates are produced, which during the storage of wine will liberate 2-AAP. The development of a robust method to analyse the content of TR and of IAA at the microgram per litre level is necessary in order to study the formation of 2-AAP precursors and of other indole compounds in wine. Must and wine are complex matrices which require careful optimisation of the methods in order to avoid interferences and produce reliable results. In particular, the low levels of IAA require a quick and

simple preparation of the sample, since almost every step of the clean-up can produce artefacts leading to important losses of the analyte.

As other possible degradation products from the same precursors, we can include tryptophol (TRO), indole and skatole. TRO is a metabolite originating from TR during the fermentation process [12]. Indole possesses a jasmine and faeces-like odour, and has been reported as a component of wine aroma with strong odour intensity [4,13,14]. Skatole is the most aromatic compound with this chemical structure — with a faecal scent — and has only recently been detected by bidimensional gas-chromatography and recognized to be present together with indole at submicrogram to microgram per litre levels in wine spoiled by 2-AAP [Rapp A., personal communication]. Indole, ethyl-indole-3-acetate and skatole have been indicated to originate mainly from IAA during the fermentation process [6]. A proper determination of TRO in neutral extracts of white wines by HPLC–UV has been reported to be not always possible, due to the presence of various overlapping impurities [15]. Only a few qualitative data have been published to date about indole in wine, and no data about skatole. An additional goal of this paper is therefore to optimise the quantification of TRO, indole and skatole.

A thin-layer chromatography (TLC) method for the analysis of indole derivatives, including TR, TRO and IAA in red and white wines was developed by Mayer and Pause [16]. More recently, a HPLC–ESI–MS–MS method allowing the quantification of IAA in grape must by isotope dilution assay has been developed [17]. This tandem mass spectrometry method is the only recent procedure published to date: it requires a triple stage quadrupole system with electrospray ionization interface, and isotope labelled internal standards. A more affordable approach by reversed-phase (RP) HPLC with fluorescence detection and clean up by an anion-exchange technique has been recently proposed for the determination of some tryptophan metabolites, including IAA, TR and TRO, in grapes, must and wine of the varietals Müller-Thurgau and Kerner [18].

The present paper describes the development and validation of a RP-HPLC fluorescence method for IAA, TR, TRO and other related indoles, where the required selectivity and sensitivity was gained

through a solid-phase extraction (SPE) clean up on a polystyrene-based polymer column. In order to choose a proper internal standard, four other indole compounds, namely indole-3-carboxylic acid, indole-3-propionic acid (IPA), indole-3-methanol and indole-2-carboxylic acid ethylester were tested together with the above mentioned TR degradation compounds.

2. Materials and methods

2.1. Standards

A solution containing ten compounds was used for the choice of the column, of the internal standard, and for the development of the conditions of separation and detection. Chemical structures and optimised chromatographic separation of these compounds are given in Figs. 1 and 2. IAA, TR, IPA, TRO, indole, indole-3-methanol and indole-3-carboxylic acid were purchased from Aldrich (Milano, Italy), indole-2-carboxylic acid ethylester from EGA-Chemie (Germany), ethyl-indole-3-acetate from Lancaster Synthesis (Lancaster, UK), and skatole from Sigma (Milano, Italy).

2.2. Other reagents

The buffer solution (BS) at pH 7.0 ± 0.05 ($20°C$) was prepared by diluting to 500 ml, with water, the potassium dihydrogen phosphate–sodium hydrogen phosphate Normex solution (final 17.34–27.2 mmol/l respectively, Carlo Erba, Rodano, Italy), MeOH was for spectrophotometry UV-FLUO (Carlo Erba).

2.3. Solid phase extraction

A stock solution containing IAA 0.5 mg/l, TRO 0.5 mg/l, TR 1.0 mg/l and IPA 0.5 mg/l in the BS was prepared to test the SPE conditions. Two different pre-packed cartridges and different eluting conditions were tested.

2.4. Test conditions

A Sep-Pak Plus C_{18} Environmental (Waters, Milford, Massachusetts, USA, 1 g) was conditioned with
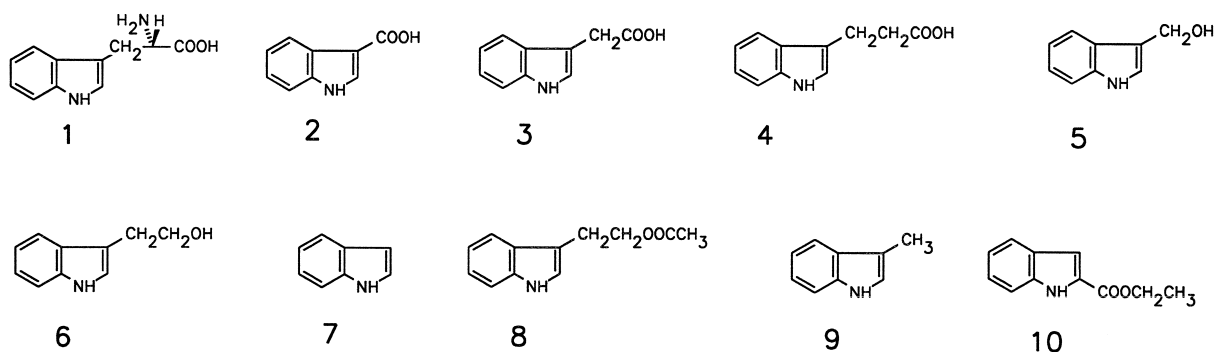
Fig. 1. Structures of the indole derivatives. (1=L-tryptophan, 2=indole-3-carboxylic acid, 3=indole-3-acetic acid, 4=indole-3-propionic acid, 5=indole-3-methanol, 6=tryptophol, 7=indole, 8=ethyl-indole-3-acetate, 9=skatole, 10=indole-2-carboxylic acid ethylester).

5 ml MeOH followed by 10 ml of BS. Five ml of stock solution was then loaded at an approx. speed of 2 ml/min. The column was washed with 10 ml of BS and the first elution was carried out with 5 ml of buffered MeOH of different strengths (2.5 to 50% v/v MeOH in BS). The second elution was carried out with 5 ml of MeOH. Both eluates were directly injected into the HPLC with fluorescence detection using column 1 with the neutral mobile phase (MP1), for the simultaneous quantification of all compounds.

A Isolute ENV+ (International Sorbent Technology, Mid Glamorgan, England, 1 g, 6 ml) was conditioned as above. Ten ml of stock solution was then loaded and the column was washed with 2×10 ml of BS. The first elution was carried out with 10
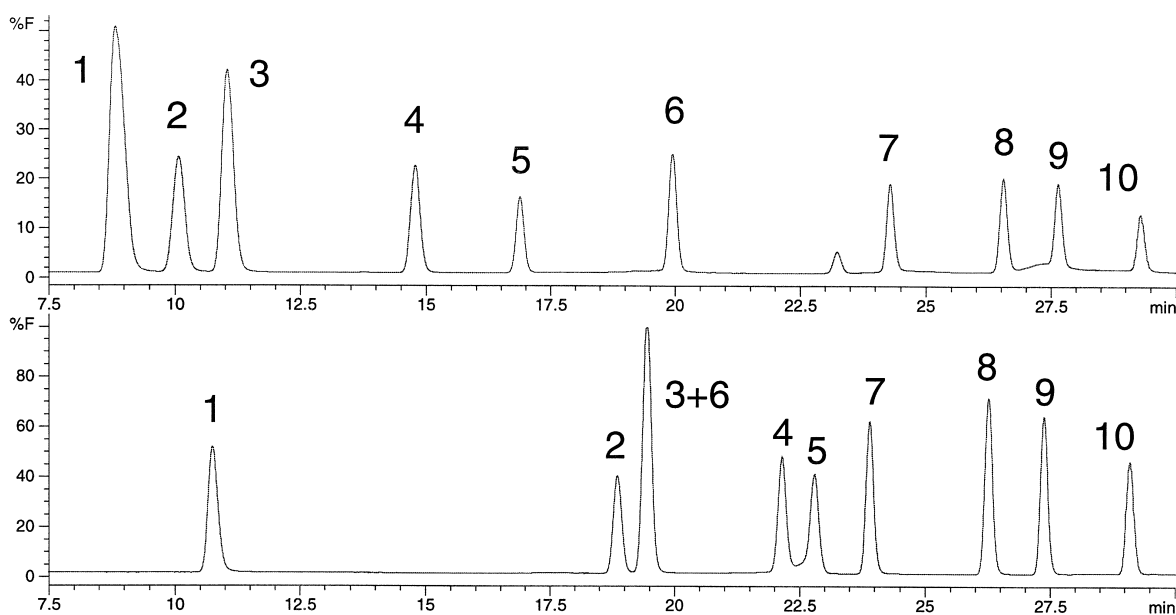

Fig. 2. Chromatographic separation of a solution of indole derivatives on a Purospher RP-18e column. Vertical axis represents the % Fluorescence. (top, neutral eluents, gain of the photomultiplier=9; bottom, acid eluents, gain of the photomultiplier=11). For the names of the compounds see Fig. 1.

ml of buffered MeOH of different polarity (10 to 100% of MeOH in BS) and the second elution with 10 ml of MeOH. The analysis was carried out under the same conditions as above.

2.5. Optimised conditions A

A Isolute ENV+ (1 g, 6 ml) was conditioned with 5 ml MeOH followed by 10 ml of 20% MeOH in BS. Ten ml of must or white wine containing IPA (Internal Standard, 200 μg/l) was neutralized to pH=7.0 by means of 5N NaOH, diluted 1:1 with BS in the case of fortified wines, and then loaded at a speed of ca. 2 ml/min. The column was washed with 2×10 ml of 20% MeOH in BS and the first elution of TR, IAA and IPA was carried out with 10 ml of 55% MeOH in BS. A second elution of TRO was carried out with 10 ml of MeOH. Both the eluates were directly injected into the HPLC. Optimal for

TR, IAA, IPA and for TRO respectively (Fig. 3, top and bottom left).

2.6. Optimised conditions B

For the additional analysis of the later eluting components (Fig. 3, bottom right), the wine sample was added to a second Internal Standard (indole-2-carboxylic acid ethylester, 1 μg/l) and processed as above up to the first elution. The second elution of TRO, indole, skatole, ethyl-indole-3-acetate and indole-2-carboxylic acid ethylester was carried out with 10 ml of CH_2Cl_2–pentane 2:1 (v/v). The second eluate was then transferred to a 15 ml flask and concentrated 20 times by means of a Vigreux condenser (A.M.S., Trento, Italy, 16×1 cm, standard joint 8 UNI cone, in water bath, 43°C) to a final volume of approx. 0.5 ml. Fifty percent MeOH in water (0.5 ml) was then added and the mixture was
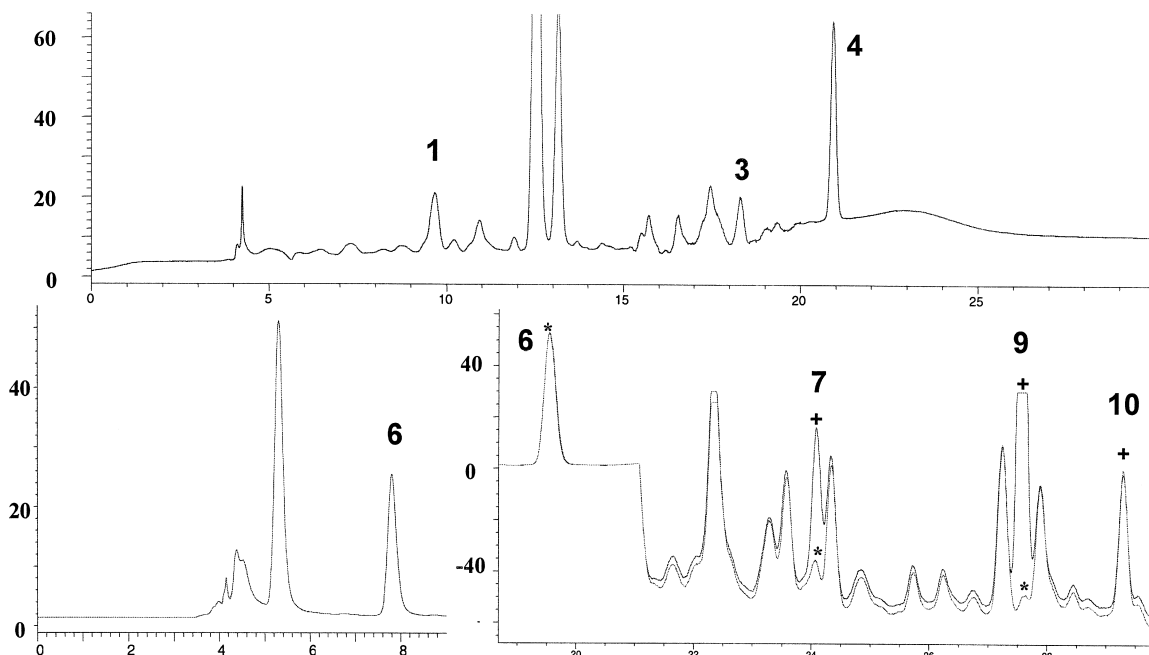

Fig. 3. Chromatographic separation on Purospher RP-18e of white wine samples, after fractionation on Isolute ENV+. Vertical axis represents the % Fluorescence at variable values of gain of the photomultiplier (PG). Gradient analysis of TR, IAA, IPA in fraction 1 (top, PG=13 to 15 min, PG=15 to 20 min, then PG=12). Isocratic analysis of TRO in fraction 2 (bottom left, PG=10) and a detail of the gradient analysis of neutral indole compounds (bottom right, PG=7 to 21 min, then PG=15). Legend: *: natural trace levels; +: wine spiked with 1 μg/l.

further concentrated to 0.5 ml before injection into the HPLC (column 1).

2.7. Chromatographic conditions

Five different columns were tested: (1) Purospher RP-18e (endcapped) 250×4 mm, 5 µm (Merck, Darmstadt, Germany); (2) Purospher RP-18, 250×4 mm, 5 µm (Merck); (3) Lichrospher 100 RP-18, 250×4 mm, 5 µm, (Merck); (4) PTH-AA 250×2.1 mm (Hewlett Packard, Waldbronn, Germany), C_{18} Hypersil ODS, 5 µm; (5) Resource RPC (Amersham Pharmacia Biotech, Uppsala, Sweden, 100×6.4 mm), a column designed for low pressure systems up to 30 bar, filled with spherical particles (15 µm diameter) of a rigid, monodispersed bead of polystyrene cross-linked with divinylbenzene.

The Hewlett-Packard (HP) 1100 was equipped with the fluorescence detector HP-1046A, operating for maximal sensitivity with excitation at 225 nm, emission at 365 nm, cut-off filter at 345 nm and volume injected 50 µl. A HP 1090L equipped with a diode array detector was also employed for the experiments requiring UV detection. The indole compounds showed maximal UV absorption at 219 and 279 nm.

Two different mobile phases (MP) were chosen. Neutral conditions (MP1), A: BS filtered at 0.22 µm, B: MeOH. Acid conditions (MP2), A: 1% acetic acid in water (pH=2.7), B: MeOH.

For columns 1 and 2, the optimised gradient both for neutral eluents MP1 and for acid eluents MP2 was: flow 0.5 ml/min, linear gradient from 25% to 100% B in 30 min, 5 min at 100% B, 5 min post-run time (Fig. 2). The isocratic TRO analysis was carried out with column 1, flow 0.5 ml/min, MP2, 60% MeOH (Fig. 3).

The columns 3–5 were compared working with MP2, linear gradient from 40 to 100% B in 30 min, 5 min at 100%, 5 min post-run time, with flow at 0.5, 0.25 and 0.4 ml/min, respectively for the three columns.

Injections at different temperatures were performed by conditioning the eluents in a thermostatic bath and connecting the column to the detector with a long capillary (0.8 m), keeping it at a constant temperature by immersion in a water bath.

2.8. Samples

The commercial wines were obtained from the winery of the Istituto Agrario di S. Michele (ISMA). The grape must samples were obtained by pressing fresh white grapes at laboratory scale. Experimental Chardonnay wines of the 1997 vintage were obtained from the Experimental Winery of ISMA and were produced with the same standardized oenological protocol [19]. The wines were produced from vines cultivated with eight different training systems, in a field divided in two lots according to different levels of soil fertility and vegetative potential (Table 2).

3. Results and discussion

3.1. Detection and temperature

The fluorescence should be preferred to the UV detection which lacks both the necessary sensitivity and selectivity for the white must and wine matrices. The intensity of fluorescence of TR and IAA in 0.1 *M* phosphate buffer at pH=7.0 was reported to decrease — as high as 5% per 1°C — with rising temperature [20]. Our results in neutral eluents MP1 for TR and IAA, whose areas were not particularly variable in the range 10–40°C, were not consistent with that of the literature. A strong variability was registered in our case only for the compounds 5, 7, 8 and 9.

Working between 5 and 40°C with the acid eluents MP2 we observed only a weak decrease of the area with rising temperature. In the worst case — for indole-3-carboxylic acid — the average decrease was 0.22% per 1°C. Control of the analysis temperature by means of specific devices does not seem to be a very important factor for the sensitivity and the precision of the analysis with MP2 in an air-conditioned laboratory.

3.2. Mobile phase and column

The resolution of TR metabolites was tested both in acid (MP2) and in neutral (MP1) eluents. A good separation of all components of the solution, including IAA — which has a pK_a=4.8 [21] — and TRO, can be obtained on the Purospher RP-18e (column 1)

with the neutral eluents MP1, where IAA shows a higher fluorescence in respect of acid conditions (Fig. 2). In wine, neutral eluents cannot be recommended because many unidentified — mostly neutral — compounds elute with or very close to IAA. In acid eluents (MP2), the presence in wine of TRO constitutes the major problem since TRO and IAA cannot be efficiently resolved (Fig. 2). TRO is present in much higher concentrations, up to 6.86 mg/l [15,22], and has approximately five times higher fluorescence than IAA. The resolutions obtained with the columns 2, 3, 5 and particularly 4 were poorer than for column 1. In particular, column 2 showed strong specific interactions of the residual silanols with the acidic compounds, especially those with one acetic or longer acid lateral chain on the indole ring.

It was therefore decided to separate IAA from TRO by SPE with a reverse phase cartridge, under neutral conditions, followed by the chromatographic analysis with column 1, in acid conditions MP2.

IPA (200 μg/l) was chosen as internal standard for the quantification of IAA and TR, since it was not reported in must or wine and — in agreement with the results of Akiyama [23] — it has a molecular structure very similar to IAA, can be efficiently monitored in the same conditions, and it elutes in a clean area of the chromatogram soon after the IAA. A second internal standard, indole-2-carboxylic acid ethylester, was suitable for the quantification of indole and skatole.

3.3. Sample clean-up

Very efficient extraction of TR, IAA, IPA and TRO from aqueous solutions can be obtained by SPE on different reversed-phase sorbents. In wine, the higher eluting strength due to the presence of ethanol must be taken into account. The extraction of indole compounds and the separation of acid (IAA and IPA) and amphoteric (TR) from neutral (TRO) compounds have been optimised on two different sorbents, the Sep-Pak Plus C_{18} Environmental (1 g), an established C_{18} material for SPE, and the Isolute ENV+ (1 g). The latter is the most hydrophobic of all Isolute non-polar sorbents, very efficient for water-soluble analytes. The sorbent is a high capacity, highly crosslinked polystyrene-based polymer column (declared specific surface area 1000 m^2/g, average particle diameter 101 μm).

The Isolute ENV+ cartridge allows a more complete extraction of all compounds from wine without the need for a dilution of the sample to lower the alcohol content, because even the more polar indole derivatives are almost completely retained up to 20% MeOH, a stronger eluent compared with the 10–14% EtOH of most wines (Fig. 4). On the other hand, with the C_{18} the TR and IAA are respectively 63.7% and 73.3% eluted with 2.5% MeOH. The elution curves demonstrate that the percentage of MeOH for recovery is lower for all constituents with C_{18}, which allows a complete recovery with 45% MeOH. On the polystyrene-based column, the total recovery of IAA and IPA can be obtained with 55% MeOH, while TRO is quantitatively retained up to 70% MeOH. With the C_{18} cartridge, 10% more MeOH in the eluent was required between the complete elution of IAA (10% MeOH) and the beginning of the elution of TRO (20% MeOH).

Taking everything into account, the quantitative extraction and fractionation of IAA and TRO can be more efficiently performed on the polystyrene-based polymer column, in the above reported optimised conditions. It must be noted that with the Isolute ENV+ cartridge, and optimised conditions, the other neutral indole compounds of the stock solution (indole, skatole, etc.) are eluting in the same fraction as TRO.

3.4. Peak identification

The identity and the purity of TR, IAA and TRO peaks monitored in wine and must were confirmed by comparing their UV spectra — obtained by means of a diode array detector — with those of authentic standards. In order to confirm the identity of the IAA peak, present at very low concentrations, it was necessary to concentrate the 55% MeOH fraction eluted from the Isolute cartridge. The 55% MeOH fraction containing IAA was exchanged into 100% MeOH, by loading it again — after a 5-fold dilution with water — on the Isolute ENV+, and then eluting it with 10 ml of 100% MeOH. By adding ca. 500 mg/l of SO_2 in the sample prior to the preparation and in the eluate, and concentrating under gentle nitrogen flow, it was possible to obtain approx. 100%
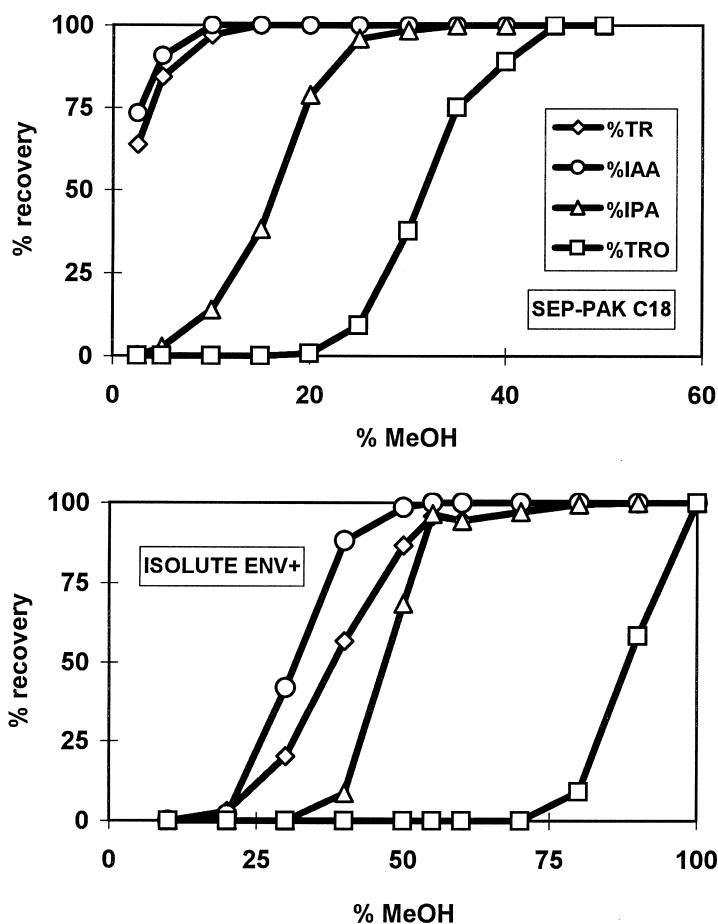
Fig. 4. Elution profiles of TR, IAA, IPA and TRO with Isolute ENV+ and with Sep-Pak Plus C_{18} Environmental cartridges.

recovery of IAA. This method of enrichment could not be applied to confirm the identity of indole and skatole peaks monitored in wines, due to their extremely low concentrations (see Fig. 3, bottom right). The proposed tentative identification of indole and skatole requires further confirmation by com-

parison with another technique, such as GC–MS or bidimensional GC.

The limits of detection (LOD, Table 1) were obtained experimentally by injection of diluted stock solutions, at a signal-to-noise ratio of 3. The sensitivity of the method for IAA analysis was consid-

Table 1
Calibration range for must and wine analysis ($N=7$) and limits of detection (at $S/N=3$)

Compound	Range (µg/l)	Calibration equation	Correlation coefficient	LOD[a]	LOQ[b]
L-tryptophan	50–1600	$y=0.995x+9.674$	0.999	8.7	29.0
indole-3-acetic acid	5–110	$y=10.397x+6.630$	0.997	2.9	9.7
indole-3-propionic acid	20–300	$y=2.620x+37.905$	0.997	1.2	4.0
tryptophol	10–520	$y=2.592x+11.819$	0.999	0.6	2.0

[a] Limit of detection, µg/l.
[b] Limit of quantification at $S/N=10$, µg/l.

ered adequate, being in wine similar to the quantification limit obtained by means of the HPLC–ESI–MS–MS method with a 100-fold concentration step during the sample preparation, and in grape must about four times lower than the reported limit [17].

A proper calibration range for the analysis of these compounds in must and wine in such conditions is given in Table 1. In order to have a better baseline and to keep within the working range of most of the normal samples, the photomultiplier gain was reduced for routine analysis of TR and TRO. The experimental values of LOD were computed again for the photomultiplier gain used for the sample analysis. For TR, LOD=5.4 μg/l in wine and LOD=7.0 μg/l in must; for IAA in wine and must, LOD=2.9 μg/l; for TRO in wine and must, LOD=0.4 μg/l; for IPA in wine and must, LOD=1.5 μg/l; for the later eluting indole compounds in wine with the 20-fold concentration step, indole LOD=0.08 μg/l, skatole LOD=0.02 μg/l, indole-2-carboxylic acid ethylester LOD=0.05 μg/l.

The distribution of the test results under repeatability conditions (as defined [24]) was estimated for the retention times and for the determination of concentration of the main indole compounds both in white wine (Pinot Blanc 1996 with 20μg/l IAA and 200 μg/l IPA added, N=11) and must samples (100 μg/l IAA, 200 μg/l IPA added, N=9). In wine, where the average recovery of IPA was 98.5%, the coefficient of variation (C.V.) was similar (4.6 to 5.2%) for IAA, TRO and TR, being 6.7% for IPA. A lower average recovery (83.6%) for IPA and a poorer C.V. (13.5%) for IAA were noticed in must, where the C.V. for IPA and TR was 3.3–3.6%. A C.V. of IAA ranging from 4.6% in wine to 13.5% in must was at least comparable with the value of 12.1% obtained with the HPLC–ESI–MS–MS method [17] and was considered acceptable for research purposes.

3.5. Sample results

The optimised HPLC–fluorescence method was applied to the analysis of Chardonnay wines produced from vines growing in fields with two different levels of soil fertility and vegetative potential (Table 2). The TR in wine ranged between 62 and 417 μg/l, while the TRO was between 38 and 2211 μg/l and IAA between 12 and 58 μg/l. By comparing the differences between the two groups of wines by means of the nonparametric Kolmogorov–Smirnov test, TRO appeared to be (p<0.005) higher in the wines produced from vines cultivated in fields with low levels of soil fertility and vegetative potential, conditions which are known to lower the levels of amino acids in must. The higher TRO levels could possibly be due both to a different starting concentration of TR in must, transformed during the fermentation through the Ehrlich mechanism [12] or to a different metabolism of carbohydrates [25] in musts with different levels of assimilable nitrogen. The present method can be applied to further investigate the origin of these compounds.

Table 2
Amounts of TR, IAA and TRO (μg/l) in some experimental Chardonnay wines produced from vines cultivated with different training systems, which are growing in an experimental field divided in two lots according to different levels (H=high, L=low) of soil fertility and vegetative potential

Training system	TR		IAA		TRO	
	L	H	L	H	L	H
Lyra (facing south)	70	124	25	42	360	566
Vertical Cordon	217	165	21	13	2211	129
Guyot	62	208	41	26	729	124
Geneva Double Courtain (facing south)	177	90	17	18	945	346
Geneva Double Courtain (facing north)	151	194	37	12	1294	41
Simple Courtain	140	417	26	20	1601	191
Spur-pruned Cordon	299	238	58	27	765	84
Spur-pruned Cordon, mechanical pruning	353	191	33	17	649	38
mean	184	203	32	22	1069	190

Acknowledgements

References

[1] A. Rapp, G. Versini, H. Ullemeyer, Vitis 32 (1993) 61.

[2] N. Christoph, C. Bauer-Christoph, M. Geßner, H.J. Köhler, Rebe Und Wein (1995) 350.

[3] U. Vrhovsek, J. Nemanic, Sodobno Kmetijstvo 31 (1998) 516.

[4] M. Geßner, H.J. Köhler, N. Christoph, C. Bauer-Christoph, R. Miltenberger, A. Schmitt, Rebe Und Wein (1995) 388.

[5] A. Rapp, G. Versini, Der Deutsche Weinbau 18 (1995) 18.

[6] A. Rapp, G. Versini, L. Engel, H. Ullemeyer, in: Innovations in Wine Technology. Microorganisms and Winemaking, Stuttgart, 11–12 May, Deutscher Weinbauverband, Bonn, 1998, p. 270.

[7] G. Ciolfi, A. Garofolo, R. Di Stefano, Vitis 34 (1995) 195.

[8] A. Rapp, G. Versini, L. Engel, Vitis 34 (1995) 193.

[9] M. Geßner, H.J. Köhler, N. Christoph, C. Bauer-Christoph, Rebe Und Wein (1996) 251.

[10] M. Geßner, N. Christoph, T. Simat, in: Innovations in Wine Technology-Microorganisms and Winemaking, Stuttgart, 11–12 May, Deutscher Weinbauverband, Bonn, 1998, p. 290.

[11] N. Christoph, C. Bauer-Cristoph, M. Geßner, H.J.S.T.J. Köhler, K. Hoenicke, Wein-Wissenschaft 53 (1998) 79.

[12] J.-C. Sapis, P. Ribéreau-Gayon, Annales de Technologie Agricole 18 (1969) 207.

[13] D.J. Stern, D. Guadagni, K.L. Stevens, Am. J. Enol. Viticul. 26 (1975) 208.

[14] P.X. Etievant, S.N. Issanchou, C.L. Bayonove, J. Sci. Food Agric. 34 (1983) 497.

[15] G.P. Cartoni, F. Coccioli, L. Spagnoli, J. Chromatogr. A 782 (1997) 219.

[16] V.K. Mayer, G. Pause, Mitteilungen Aus Dem Gebiete Der Lebensmitteluntersuchung Und Hygiene 57 (1966) 147.

[17] B. Dollmann, E. Richling, M. Herderich, H. Köhler, A. Schwab, A. Schmitt, P. Schreier, Vitis 36 (1997) 97.

[18] K. Hoenicke, T.J. Simat, H. Steinhart, N. Christoph, H.J. Köhler, A. Schwab, in: Tryptophan, serotonin and melatonin: basic aspects and applications, G. Huether, W. Kochen, T.J. Simat, H. Steinhart (eds.), Plenum Press, New York, 1999, in press.

[19] M. Bertamini, U. Malossini, G. Nicolini, C.R. G.E.S.Co. N.10, Changins, Suisse, 26–28 May, 1998, p. 180.

[20] G.G. Guilbault, Practical Fluorescence, Marcel Dekker, New York, 1973.

[21] G. Sandberg, A. Crozier, A. Ernstein, B. Sundberg, H.F. Liskens, J.F. Jackson, in: High Performance Liquid Chromatography in Plant Sciences, Springer-Verlag, Berlin, 1987, p. 72.

[22] L. Nykänen, E. Puputti, H. Suomalainen, J. Inst. Brew. (1965) 24.

[23] M. Akiyama, N. Sakurai, S. Kuraishi, Plant Cell Physiol. 24 (1983) 1431.

[24] AA.VV., Statistical Methods. ISO Standards Handbook 3., Genève, Swiss, 1989, p. 412.

[25] G. Reazin, H. Scales, A. Andrease, J. Agric. Food Chem. 18 (1970) 585.