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Journal of Chromatography B, 702 (1997) 103–110

JOURNAL OF
CHROMATOGRAPHY B

Gas chromatographic analysis of resveratrol in plasma, lipoproteins and cells after in vitro incubations

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Received 24 December 1996; received in revised form 14 July 1997; accepted 14 July 1997

Abstract

Resveratrol is a trihydroxystilbene present in certain red wines. It may play a role in the inhibition of lipoprotein oxidation and platelet activity. We have developed the first method to measure resveratrol in animal and human samples and to study its incorporation in vitro. After adding epicoprostanol as an internal standard, samples are subjected to lipid extraction in the presence of antioxidant and under dim light to minimize both denaturation and isomerization of the *trans*-resveratrol to the *cis*-form. Extracts were purified by cold acetone precipitation and the resveratrol-containing acetone phase was evaporated under nitrogen. The resveratrol was analyzed as a trimethylsilyl derivative by capillary gas chromatography which resolved the *cis*- and *trans*-resveratrol (6.6 and 12.9 min, respectively). Analyses of samples spiked with pure *trans*-resveratrol (0.1 to 10 μg) indicated that the method was specific and gave excellent linearity and recovery (96.8%) with a high reproducibility (coefficient of variation: 3.3%). The detection limit was about 50 ng/ml. Applications show that resveratrol was incorporated into blood cells and lipoproteins after in vitro incubations with plasma, lipoproteins and cells. © 1997 Elsevier Science B.V.

Keywords: Resveratrol; Erythrocytes; Platelets; Phytosterols

1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene, Fig. 1) is a compound found both free and as glycosides in two isomeric forms in a number of plant species [1–4]. Although, *cis*-resveratrol has been clearly identified most of the investigations focused only on the *trans*-isomer. The synthesis of resveratrol has been studied in vines and it is enhanced by injury, light and fungal infection [5–8]. Resveratrol is thus considered as a phytoalexin and part of a general defence mechanisms against physical or fungal stresses. Recently, Siemann and Creasy [9] reported that resveratrol was

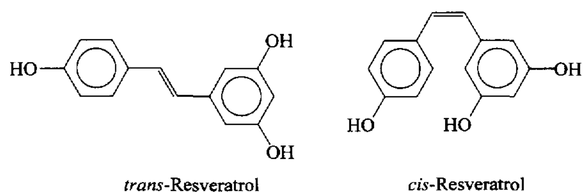

Fig. 1. Chemical structures of *trans*- and *cis*-resveratrol (3,5,4′-trihydroxystilbene).

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PII S0378-4347(97)00383-6

present in wines and this stimulated research programs dealing with its pharmacological properties. Resveratrol has been identified in Japanese folk medication and more recently found to decrease lipid deposition in liver [9], to inhibit blood platelet aggregation [10–12] and lipid peroxidation [13,14]. Resveratrol might be one of the compounds responsible for the decreased mortality from coronary heart disease associated with wine-drinking populations [15,16]. However, this idea is open to discussion because of the low concentration of resveratrol in wines (2–5 mg/l) and the relatively high concentrations of this compound (1–5 mg/l) necessary to observe its effects in vitro.

A number of methods have been published to measure resveratrol in plant products such as leaves, roots, berries or wine [7,8,17–20]. Most of these methods use HPLC with UV detection or GC with a flame ionisation detector or mass spectrometric detection. To our knowledge, no method has been reported so far to determine resveratrol in animal or human biological samples. We report in the present paper, the first assay of the resveratrol incorporated into cell, plasma and low density lipoproteins (LDL) after in vitro incubations.

2. Experimental

2.1. Chemicals and reagents

The *trans*-isomer of resveratrol was synthesized and kindly obtained 99.6% pure from Drs. Jeandet and Bessis [6] from the Institut Universitaire de la Vigne et du Vin (Dijon, France). A stock solution of 1 mg/ml in ethanol was maintained at −20°C in a dark container. The *cis*-resveratrol was obtained through UV-photoisomerization (254 nm, 2–4 h) of the standard solution of *trans*-resveratrol. Pyridine (dried over molecular sieves) was from Merck and a mixture of N,O-bis (trimethylsilyl)acetamide–trimethylchlorosilane–trimethylsilylimidazole (BSA–TMCS–TSIM, 3:2:3, v/v) was purchased from Supelco. The internal standard, epicoprostanol (5β-cholestan-3α-ol) was from Sigma and was 95.6% pure; the impurity was coprostanol and has been taken into account for the calculations. 2,6-Di-*tert*.-butyl *p*-cresol (BHT) was from Fluka. Dichloromethane, methanol as well as other solvents of analytical grade were obtained from Merck or Prolabo.

2.2. Biological samples

After an overnight fast, blood samples were taken from volunteers by venipuncture into tubes containing disodium EDTA (1.5 mg/ml), and the plasma was separated in a cold centrifuge. Low density lipoproteins (LDL, 1.019–1.055 g/ml) were isolated by sequential ultracentrifugation as in previous studies [21]. Pooled LDL were dialyzed against phosphate buffered saline (PBS) (pH 7.4) containing 50 μ*M* EDTA. They were sterilized by passage through a Millipore filter (0.22 μm) and stored under argon at 4°C in the dark for no longer than 15 days. Proteins were measured using bicinchoninic acid [22] and bovine serum albumin as standard.

For animals samples, blood was removed as in previous studies [23] from the jugular vein of overnight fasted rats, into plastic syringes containing one volume of anticoagulant (38 m*M* citric acid; 75 m*M* sodium citrate; 136 m*M* glucose) for four volumes of blood. The platelet-rich plasma (PRP) was obtained after centrifugation (220 *g*, 10 min) and further centrifuged (900 *g*, 10 min) to separate platelets from platelet-poor plasma (PPP). The platelets pellet was washed, counted with a Coulter counter ZM (Coultronics) and resuspended ($1\cdot10^9$/ml) in a Ca^{2+}-free Tyrode's buffer (pH 6.8) containing 149 m*M* sodium chloride, 2.6 m*M* potassium chloride, 9.5 m*M* sodium bicarbonate, 5.5 m*M* glucose, 0.5 m*M* dihydrogen orthophosphate and 0.6 m*M* magnesium chloride.

Red blood cells were washed twice with saline and resuspended in 15% hematocrit.

2.3. Sample processing

The whole procedure was carried out in dim light to reduce the photoisomerization of *trans*-resveratrol to the *cis*-isoform. After addition of epicoprostanol (5 μg) as internal standard, total lipids were extracted. For plasma (0.2 ml) and platelets (10^9) extraction was carried out using the method of Folch et al. [24] with CH_2Cl_2–CH_3OH (2:1, v/v). For erythrocytes, the method of Rose and Oklander [25]

(0.1–0.4 ml packed cells) was used with 0.05% BHT-containing CH_2Cl_2 instead of chloroform, and isopropanol (3:4, v/v). The lipid extracts were evaporated under nitrogen gas and cold acetone (1 ml, −20°C) was added to the dried residue, vortex-mixed and sonicated for 20 s and left for at least 30 min on ice. Polar lipids were precipitated and after centrifugation (2000 *g*, 10 min, 4°C), the resveratrol-containing acetone phase was transferred to conical glass tubes. The precipitate was washed with cold acetone (0.5 ml) and centrifuged again. The acetone phases were evaporated under a stream of nitrogen and the extract was derivatized to trimethylsilyl ether (TMS) by heating 10 min at 50°C with the mixture of BSA–TMCS–TSIM in pyridine (30:70, v/v). When large amounts of sterol esters were expected in samples such as in hyperlipemic plasmas, the evaporated acetone phase was saponified with 0.5 ml cold 1 *M* potassium hydroxide, flushed with nitrogen and allowed to proceed at room temperature for 3 h. The resveratrol was then extracted twice with dichloromethane (2 ml) and derivatized after evaporation as previously described.

2.4. Gas chromatographic analysis

An aliquot of the derivatized mixture was injected in the chromatograph (DI200, Delsi, France) equipped with a all-glass falling needle injector (Spiral, Dijon, France), a flame ionisation detector and a bonded capillary column (OV 1701, 0.1-μm thickness, 25 m×0.32 mm I.D.). Conditions were: injector 270°C, detector 300°C, oven set at 240°C and programmed to reach 275°C at a rate of 2°C/min with helium velocity set at 0.20 m/s. Data acquisition and quantification were done with the help of the internal standard and a computerized terminal (Chroma Software, Biosystems, France). In some cases, samples were analyzed by GC–MS using a HP5971A mass detector connected with a HP5890 gas-chromatograph (Hewlett Packard).

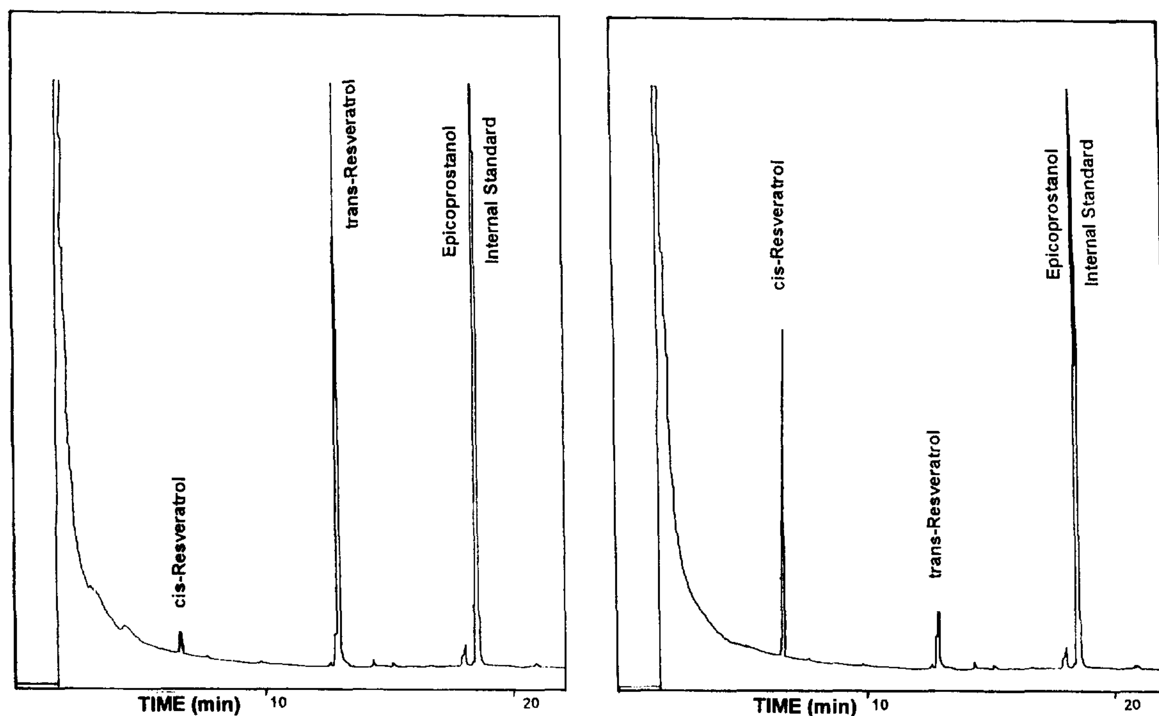

Fig. 2. Gas–liquid chromatograms of trimethylsilyl ethers of *trans*- (left) and *cis*-resveratrol (right) standards. The *cis*-form was obtained by UV photoisomerization of *trans*-resveratrol. Epicoprostanol was used as an internal standard.

Table 1
Analysis of sterols and resveratrol isoforms by capillary gas chromatography: retention times of their TMS derivatives

Compound	Relative retention time	
	OV 1701	SE 30
Epicoprostanol	1.000	1.000
cis-Resveratrol	0.354	0.320
trans-Resveratrol	0.695	0.570
Cholesterol	1.168	1.091
Campesterol	1.330	1.180
β-Sitosterol	1.521	1.306

Retention times are expressed relative to the internal standard, epicoprostanol which eluted at 18.42 min and 14.63 min on OV1701 and SE30 capillary columns, respectively. Chromatographic conditions as detailed in Section 2.4.

3. Results and discussion

Preliminary results of derivatization and chromatographic analysis of standard of resveratrol have shown that improved sensitivity and resolution were achieved using TMS ether compared with underivatized resveratrol. Consequently we decided to analyze resveratrol as TMS ether. Fig. 2 shows typical chromatograms obtained with standards and the position of the *cis*-, *trans*-resveratrol and epicoprostanol peaks. Concerning our choice for an internal standard, diethylstilbestrol, once derivatized, eluted close to resveratrol and could be present in some human samples whereas *cis*- and *trans*-stilbene had no silylable hydroxyl group to be fully representative

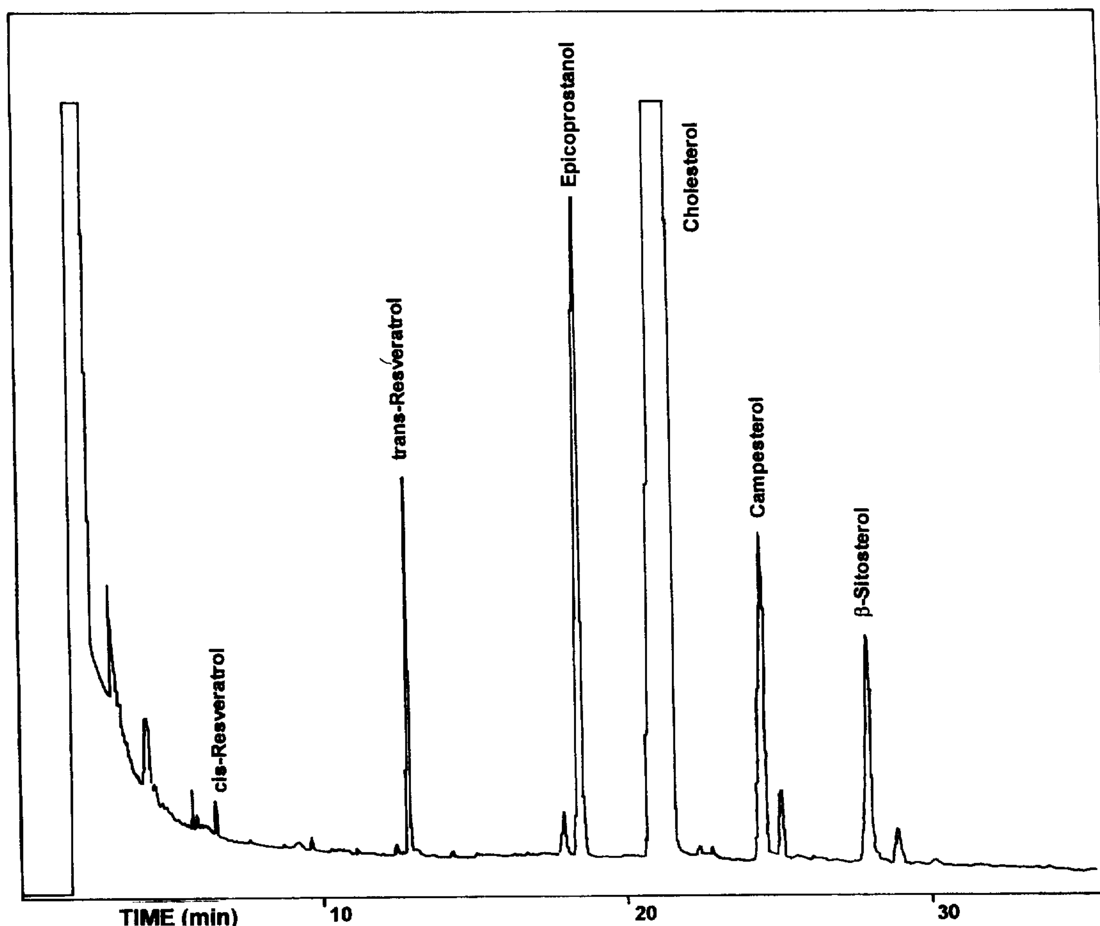

Fig. 3. Chromatogram of the analysis of *trans*-resveratrol added to erythrocytes. *trans*-Resveratrol (5 μg) has been added to homogenates of rat erythrocytes and analyzed using our described method.

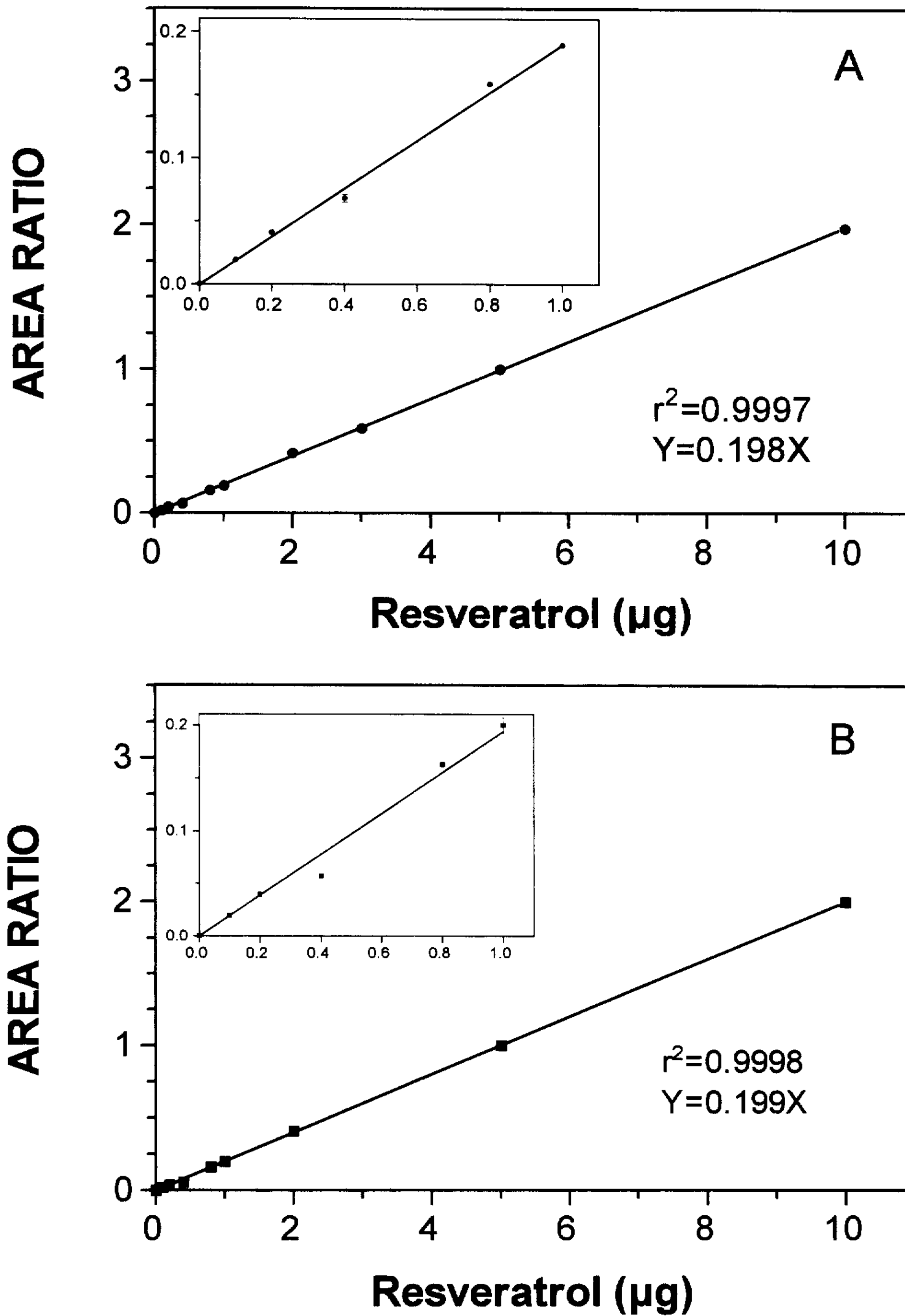

Fig. 4. Calibration and linearity of various *trans*-resveratrol amounts added to rat erythrocytes (A) or rat plasma (B). Endogenous resveratrol was not detectable. Results of resveratrol recovered were expressed as the area ratio of resveratrol to epicoprostanol (internal standard). Insets represent detail of the values for the range up to 1 μg resveratrol.

of the whole process. Consequently, we chose epicoprostanol as it is easily available. In our conditions using OV 1701 column, *cis*- and *trans*-resveratrol eluted at 6.57 and 12.89 min, respectively. Table 1 shows retention times relative to epicoprostanol on another commonly used less polar column (SE30, 25 m).

3.1. Extraction procedure, specificity and recovery

To test our extraction procedure, red blood cell homogenates obtained by freezing and thawing and devoid of endogenous resveratrol were used. They were spiked with a known amount of *trans*-resveratrol and extracted according to our procedure with epicoprostanol as internal standard. We found that resveratrol chemically behaved like a sterol as it was recovered in the acetone phase which also contained sterols. A typical chromatogram of resveratrol analysis in red cells is shown in Fig. 3. Beside cholesterol which is the major sterol, minor phytosterols such as campesterol and β-sitosterol were identified. Further characterization of resveratrol was achieved by UV photoisomerization which resulted in a decrease of the peak eluting at 12.89 min (*trans*-resveratrol) and a subsequent enlargement of the peak at 6.57 min (*cis*-form). In addition, analyses using GC–MS revealed that the mass spectra of the two peaks were identical and exhibited the following m/z ions: 444 (relative abundance, 100%), 429 (6.4%) and 371 (2.5%) corresponding to the molecular ion M^+, M−15 and M−73, respectively. In the 0.1–10 μg concentration range, the recovery of the *trans*-resveratrol relative to the internal standard averaged 96.8%. This high recovery was obtained using our sample purification which was based on acetone precipitation. Preliminary attempts carried out with solvent extraction (ethylacetate) such as those used for plant samples [7,19] were unsuccessful because of high losses due to the formation of emulsions. This resulted in a far lower yield. So did when the treatment in the ultrasound bath was omitted.

3.2. Calibration, linearity and reproducibility

Our calibration studies were carried out by spiking a pool of erythrocytes and plasma with various concentrations of *trans*-resveratrol (0.1–10 μg). Epicoprostanol was added to all the samples. Samples were processed as indicated in Section 2.3, including extractions according to Rose and Oklander [25] for erythrocytes and to Folch et al. [24] for plasma. The resulting calibration curves are shown in Fig. 4. They indicate that, in the respective concentration ranges, the curves obtained for erythrocytes (A) and plasma (B) are linear. The precision of the overall processing was determined by measuring the resveratrol concentration added to a pooled red cell homogenate and divided into several aliquots. Under our conditions, a precision of 3.31% (coefficient of variation, C.V.%) for a mean value of 3.24 μg/ml ($n=12$). The detection limit was found to be 50 ng/ml on the basis of a signal-to-noise ratio of 3. This limit might be further decreased by a factor of five either by repeated injections onto the needle of the injector (falling needle injector) and/or by using selective ion monitoring (SIM) of the molecular ion at mass 444 (M^+). Altogether, these results indicate that due to the high recovery achieved and to the introduction of an internal standard, our method is very reproducible.

3.3. Applications

Discrepancies between the biological activities of resveratrol and its concentration can be documented. As for other polyphenols, this is probably due to its

Table 2
Analysis of resveratrol after in vitro incubation with blood cells and LDL

	trans-Resveratrol
Human erythrocytes	2.29±0.38 μg/10^9
Rat erythrocytes	2.47±0.51 μg/10^9
Rat platelets	0.49±0.28 μg/10^9
Human LDL	0.87±0.22 μg/mg protein

Erythrocytes (1.6×10^9) or platelets (10^9) were incubated with 5 μg *trans*-resveratrol for 15 min at room temperature. Cells were then washed twice to remove excess resveratrol with buffer and extracted for resveratrol assay. LDL (0.5 mg/ml) were incubated with 4 μg/ml *trans*-resveratrol for 30 min at room temperature and after washing and filtration (2×) through Millipore filters (cut off 30 kDa) to remove unbound resveratrol, LDL-bound resveratrol (retained on the filter) was assayed using the proposed procedure. No endogenous resveratrol was detectable in the control incubations. Values represent means±S.D. of 6–8 different measurements.

highly lipophilic nature. This would result in an increased binding which could be time and concentration dependent. Our method was used to measure the resveratrol present in animal or human samples which incorporated into cells after incubation.

When blood cells such as erythrocytes or platelets were incubated with 5 μg resveratrol solubilized in ethanol (maximum final concentration: 0.05%), after washing for removal of excess resveratrol, our present method allowed us to precisely quantitate the resveratrol incorporated in these blood cells (Table 2, Fig. 5). We found that rat and human erythrocytes bound resveratrol in similar amounts.

Resveratrol has been reported to inhibit copper-mediated LDL oxidation [13]. Recently, it has been proposed that the binding of resveratrol to the lipoprotein particles improved its antioxidant activity [26]. We have therefore analyzed the resveratrol content after incubation with LDL using our method. After re-isolation of the incubated LDL to remove excess resveratrol, we found that a significant amount of resveratrol did bind to LDL (Table 2). This might account for the putative beneficial effect of resveratrol since LDL is widely documented to become rendered atherogenic by oxidation.

However, it is difficult to propose that the concentrations of resveratrol tested in these in vitro

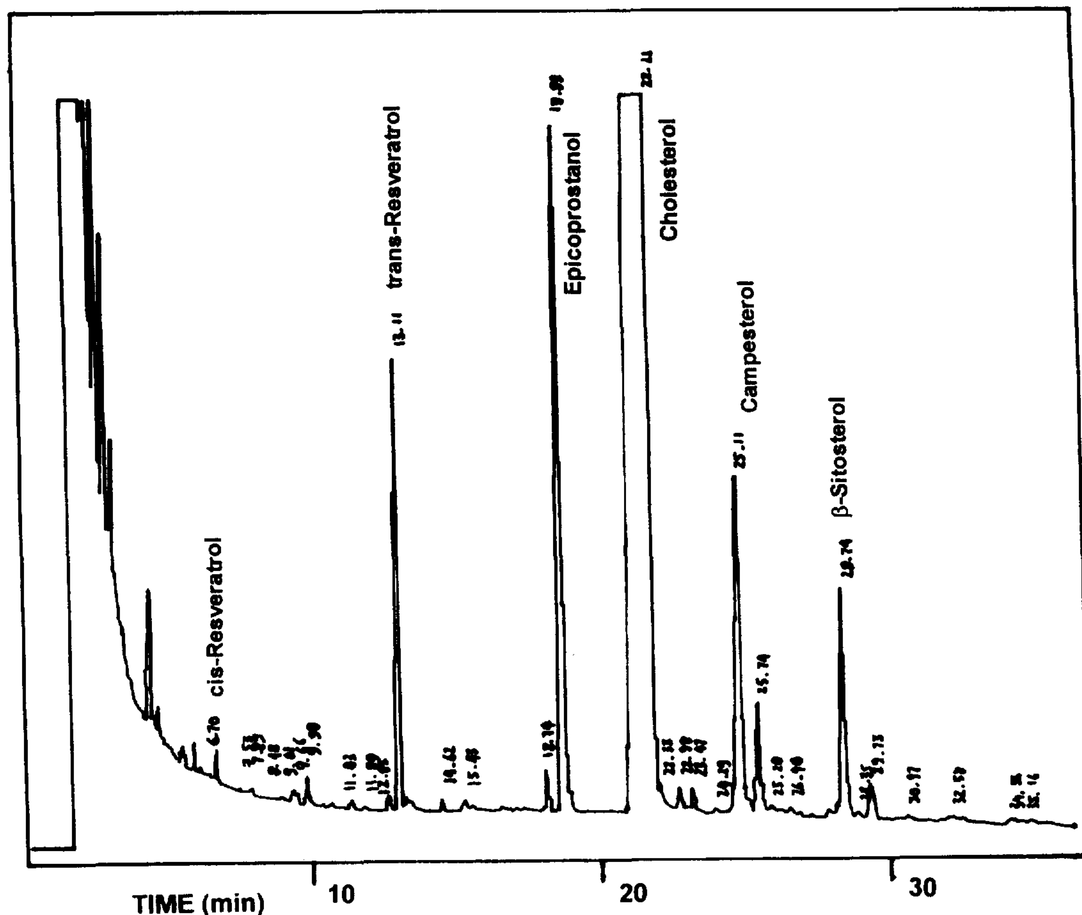

Fig. 5. Analysis of resveratrol after incubation with erythrocytes. This chromatogram shows a representative analysis of incorporated resveratrol following an incubation of rat erythrocytes with 5 μg *trans*-resveratrol for 15 min (see Table 2). After incubation, erythrocytes were washed twice with phosphate buffer and extracted according to the described protocol. Quantitation using the internal standard epicoprostanol indicated a total concentration of resveratrol of 2.65 μg/10^9 cells for this particular sample.

experiments which are sub-pharmacological ones, might be reached in vivo following a reasonable wine intake. Indeed, assuming that all the resveratrol of a 300-ml drink of red wine (high approximation: 1.8 mg) will be recovered in 5 l blood, the resulting concentration would be approximately 3.6 μg/10 ml which is about ten times less than the reported inhibitory concentrations for oxidation or platelet activity. Consequently, although such high circulating concentrations may be acutely obtained in the post-prandial state, further investigations, particularly dealing with partition and bioavailability of resveratrol merit investigation, possibly with the help of our method.

4. Concluding remarks

Our method is the first one dealing with analysis of resveratrol in animal or human samples. It is simple to handle because extraction is easily carried out without the use of expensive solid-phase extraction columns or solvents such as ethylacetate or diethylether which often lead to stable emulsions which contributes to obtaining lower yields. Moreover, diethylether often contains traces of peroxides which may oxidize antioxidant including resveratrol. Furthermore, there is no lengthy purification steps which might denature resveratrol as stilbene are unstable in light and sensitive to oxidation in air. The specificity of GC (or GC–MS) allows assay of the *cis*- and *trans*-isoforms of resveratrol in the same chromatographic run and achieves a high sensitivity. This could not be reached using HPLC with UV detection.

In summary, the procedure described in the present paper is simple, efficient and accurate. It allows one to routinely extract and analyze resveratrol isoforms in biological samples of animal and human origin in a specific and quantitative manner after in vitro incubations.

Acknowledgments

This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), the Conseil Régional de Bourgogne and the Université de Bourgogne.

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