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Quantitative determination of phenolic diterpenes in rosemary extracts
Aspects of accurate quantification

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Abstract

Practical challenges related to accurate quantification of carnosic acid (CA), carnosol (CAR) and other phenolic diterpenes in extracts of rosemary leaves (*Rosmarinus officinalis* L.) are presented and discussed. Primary standard material of CA is isolated from rosemary extracts by preparative chromatography with a purity of 98% or higher. The response factors of CAR relative to CA, at 230 and 280 nm, have been estimated to be 0.92 and 1.36, respectively. The stability of pure CA and CAR, dissolved in methanol, dimethyl sulfoxide (DMSO), DMSO–acetonitrile (10:90) or ethyl acetate–acetonitrile (10:90) and stored at room temperature in the autosampler, is presented. Pure CA dissolved in DMSO is stable for several days, while CAR showed significant degradation within a few hours in all solvents tested. The lack of stability of standards results in practical difficulties with calculating reliable response factors. A correction procedure is presented and documented. A CAR calibration solution was analysed six times for purity during 30 h of storage, while the purity changed from 95 to 70%. Applying this correction procedure resulted in a relative standard deviation on the average response factor of 0.7% ($n=6$). CA has been dissolved in methanol and stored in clear and amber glass vials, respectively. The solution stored in amber vials degraded faster that in clear vials. The high content of Ti and Fe ions in amber glass seems to catalyse the degradation of CA. In contrast to solutions of pure CAR and CA, their stabilities in solutions of rosemary extracts are fine. A standard addition experiment, covering a time interval of 21 h, resulted in recoveries of CAR and CA of 100 and 96%, respectively.

Keywords: *Rosmarinus officinalis*; Terpenes; Carnosic acid; Carnosol; Phenolic compounds

1. Introduction

Extracts of rosemary (*Rosmarinus officinalis* L.) leaves have been recognised to possess significant antioxidant properties due to the content of phenolic diterpenes. The major phenolic diterpenes are carnosic acid (CA) and carnosol (CAR) (Fig. 1). Other phenolic diterpenes like rosmanol, epirosmanol and methoxyepirosmanol are often present in small concentrations. These minor components are, at least to some extent, the result of the degradation of CA [1–3], but they still possess antioxidant activity [4,5].

Rosemary extracts are of commercial interest to the food industry as a source of natural antioxidant. The quality as antioxidant and the price of commer-

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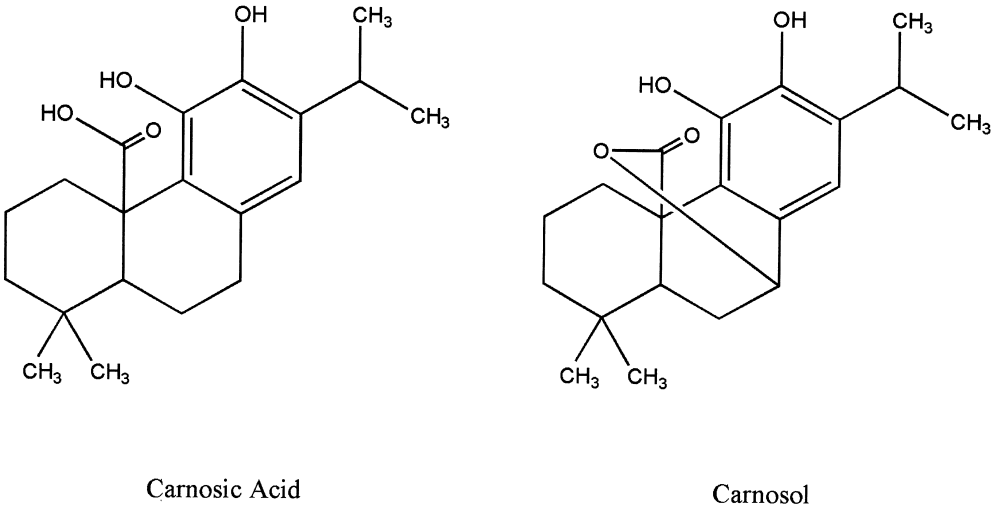

Fig. 1. Chemical structures of carnosic acid and carnosol.

cial rosemary extract is highly correlated to the content of primarily CA and secondly to the total content of phenolic diterpenes including CAR. Accurate quantitative determinations of phenolic diterpenes are therefore of great importance from the commercial aspect. These results are based on reliable reference material, which is used for calibration. As the commercial availability of reference materials is limited, it must be isolated from rosemary extracts by preparative chromatography [5]. In practise, not all reference components can be isolated and tentative quantification, based on, e.g. CA calibration, is the only practical solution. CA is the most abundant phenolic diterpene in rosemary extract and is therefore the clear choice of reference component. Several authors have used CA [5–8] and CAR [9] as reference components for quantitative determinations of phenolic diterpenes.

The method of using a single reference component is precise but may be inaccurate. The reference component and the other components may have different absorption coefficients (response factors) at the detection wavelength. These differences determine the inaccuracy introduced. As the absorption coefficient is a function of the wavelength, the choice of detection wavelength influences the inaccuracy. Careful choice of the detection wavelength can minimise the inaccuracy by choosing a wavelength at which the response factors of the individual components are known to be close to each other. Many authors use 230 nm as detection wavelength [2,5,8,10], but 280 nm [11], 284 nm [12] and 285 nm [13] as detection wavelengths are also reported. Knowledge of the response factor ratios between the reference component and the other components will further minimise the inaccuracy. In the results presented, the response factors of CA and CAR at 230 and 280 nm are compared and discussed.

One other aspect of accurate quantification is the stability of reference solutions. Phenolic diterpenes from rosemary, especially CA, are considered unstable in solution in the presence of oxygen [2,10]. In quality control environments, it is important that methods can be automated. To make automated quantitative analyses, solutions of standards and unknowns must be stable for several hours, preferably at least 1 day, i.e. solutions must be stable to ‘‘survive’’ several hours in an autosampler.

In the results presented, the following items are discussed: (a) The determination of the purity of chromatographically purified reference material. (b) The stability of solutions of pure CAR and pure CA as a function of time, solvent choice and type of glassware, and the correction for the observed degradation during storage. (c) The recovery of CAR and CA from spiked rosemary extract.

2. Experimental

2.1. Materials

Commercially available rosemary extract (powder) was used as test material. The chromatogram of the rosemary extract test material is shown in Fig. 2. The approximate contents of CAR and CA were 9 and 17%, respectively. Primary standards of CA were prepared from rosemary extracts (ca. 65% phenolic diterpenes) by preparative liquid chromatography (LC) (Instrumentals and chromatographic conditions). CAR (95%) was a gift from Cultor Food Science, New York, NY, USA.

The water used was Milli-Q quality. Acetonitrile (ACN), methanol (MeOH), hexane, acetone and ethyl acetate were all HPLC grade from Lab Scan, Dublin, Ireland. Dimethyl sulfoxide (DMSO) was from Fluka No. 41640 and trifluoroacetic acid (TFA) from Merck No. 808260.

2.2. Instrumentals

The preparative HPLC system consisted of a Waters PrepLC 4000 System (Milford, MA, USA) equipped with a Rheodyne 7125 injection valve (5 ml sample loop). The column was a Luna C_{18} (2), 5 μm, 250×21.2 mm I.D. (Phenomenex, Torrance, CA, USA). The preparative flow was split between the detector and the fraction collector using an ACURATE flow splitter (1:1000, Catalogue No.: ACM-01-10-CR), LC Packings (Amsterdam, Netherlands). A Waters 510 isocratic pump served as make-up pump for the split flow to the detector. The detector was a Waters 486 UV–Vis spectrophotometer. The fraction collector was an LKB 2111 MultiRac (LKB/Pharmacia, Sweden). The detector signals were collected by TurboChrom data management system (Perkin-Elmer Instruments, Shelton, CT, USA).

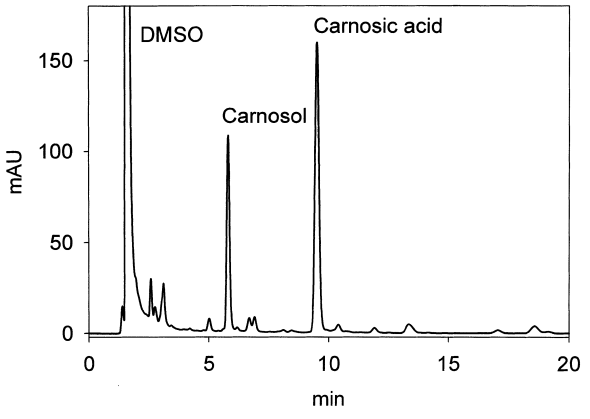

Fig. 2. The chromatogram of rosemary extract (9% CAR, 17% CA) is recorded at 230 nm. The sample amounts injected correspond to 240 ng CAR and 460 ng CA. The chromatographic conditions are described in the Experimental section.

The analytical high-performance liquid chromatography (HPLC) system was an Agilent Technologies 1100 Series system (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump, an autosampler, a thermostated column compartment and a photodiode array detector. All modules and data collection was controlled by Agilent Chemstation software. The column was a Zorbax SB-C_{18}, 3.5 μm, 150×3.0 mm I.D. (Zorbax, Agilent Technologies, Palo Alto, CA, USA).

2.3. Chromatographic conditions

The mobile phase, for the preparative separations, consisted of 350 ml water, 650 ml ACN and 0.5 ml TFA. The flow-rate was 25 ml/min. Rosemary extract (1 g) was dissolved in methanol (10 ml). The injection volume was 2 ml (partial loop filling). The make-up solvent for the flow splitter was methanol. The make-up flow-rate was 1.0 ml/min. The detector was set at 230 nm.

The collected fractions of CA, eluting from 7.9 to 9.6 min, were evaporated under reduced pressure (max. 40 °C) to remove ACN. The remaining water and TFA were removed via freeze–drying. The raw purified primary standard of CA was re-crystallised from acetone–hexane. The purity of CA was 98% or higher.

The analytical mobile phase consisted of solvent A (400 ml water+600 ml ACN+1.5 ml TFA) and solvent B (1000 ml MeOH+1.5 ml TFA). The flow-rate was 0.42 ml/min. The samples (standard or rosemary extract) were dissolved in selected solvents (Section 3). The injection volume was 5.0 μl. The separation was accomplished by running 100% solvent A for 30 min. From 30 to 31 min 100% solvent B was introduced to rinse the column. Then, 100%

solvent B was kept from 31 to 36 min. From 36 to 40 min, the gradient returned to 100% solvent A. The column equilibrated at 100% solvent A from 40 to 50 min. The column temperature was 45 °C. Two detector wavelengths were set: (wavelength; bandwidth) 230; 4 nm and 280; 4 nm both with reference wavelength at 550; 80 nm. Spectral range was from 200 to 600 nm.

2.4. Solvent pre-treatment

Prior to use, all solvents were degassed with vacuum and ultrasonic treatment to exclude dissolved oxygen from the solvents followed by sparging with nitrogen. The headspaces of all solutions/flasks were purged with nitrogen to exclude oxygen.

2.5. Preparation of standards and samples

CA (5 mg) was dissolved in DMSO (5 ml) and diluted with ACN to volume (25 ml). Further dilutions were obtained by dilution with ACN. Blank samples were prepared from DMSO (5 ml) diluted to volume (25 ml) with ACN.

Rosemary extracts (25 mg) were dissolved in DMSO (5 ml) and diluted to volume (25 ml) with ACN.

For standard addition, a sample of rosemary extract (25 mg) is weighed into 50 ml volumetric flasks. Freshly dissolved standard of CAR (0.4 mg/ml DMSO) or CA (1.1 mg/ml DMSO) is added (1.0 ml). DMSO is added to 5 ml and the sample is dissolved. The flask is filled to volume with ACN. Additional spiked samples are prepared similarly by adding 2.0 and 3.0 ml standards, respectively. Note that the samples are spiked with only one component at a time.

3. Results and discussion

3.1. Standard materials

Primary standard of CA was obtained from rosemary extracts by preparative chromatography. The extract used, was specified having a phenolic diterpene content of about 65%, of which CA was the major component. Suspending the extract in hexane seems to dissolve most of the green/brown coloured components, leaving an off-white precipitate rich in CA. Introducing this purification step, improves the throughput of high purity CA in the chromatographic step.

3.2. Estimation of the purity of reference materials

The purities of both CA and CAR were estimated by peak area-percentage at 230 nm. The peak area-percentage is calculated as: ‘‘The peak area-ratio between the peak-of-interest (CAR or CA) and the sum of all detected peaks (excluding the solvent peak and other peaks present in a solvent blank) multiplied by 100’’. The purity is equivalent to the calculated peak area-percentage.

3.3. Quantification at 230 vs. 280 nm

The error introduced using peak area percentage for purity determination is dependent on the differences between the response factors of the components present and the reference component. The response factors are a function of the detection wavelength. The choice of detection wavelength may therefore be of significant importance to minimise the error on the purity determination.

The relative response factors (RRF) at 230 and 280 nm have been estimated for CA and CAR. When RRF (CA, 230 nm) is set to 100, the other values becomes RRF (CAR, 230 nm)=92.1, RRF (CA, 280 nm)=14.5 and RRF (CAR, 280 nm)=19.7, respectively. The response factor ratios (RF-ratios) at 230 and 280 nm, between CAR and CA, can then be estimated: RF-ratio (CAR/CA, 230 nm)=0.92 and RF-ratio (CAR/CA, 280 nm)=1.36. All values are obtained after correction for purity as described below in Section 3.4. For quantitative analyses, it may be convenient to use only CA as calibration component. Applying the relative response factor for CAR, a more accurate quantification of CAR is obtained.

For quantitative analysis, CA is best detected at 230 nm although it is not a maximum absorbance wavelength but merely a shoulder at the spectrum curve. The absorption coefficient is about six times larger compared to 280 nm (Fig. 3). One should normally avoid a wavelength where the slope of the

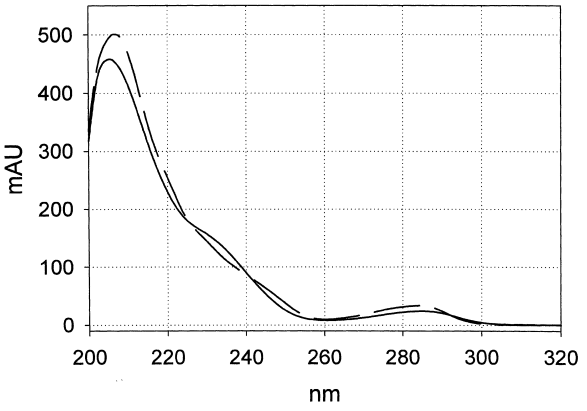

Fig. 3. UV-spectra of CA (solid) and CAR (dashed). The spectra are scaled to same concentration. The extinction of CAR is lower than CA at 230 nm (92%), while the extinction of CAR is higher than CA at 280 nm (136%).

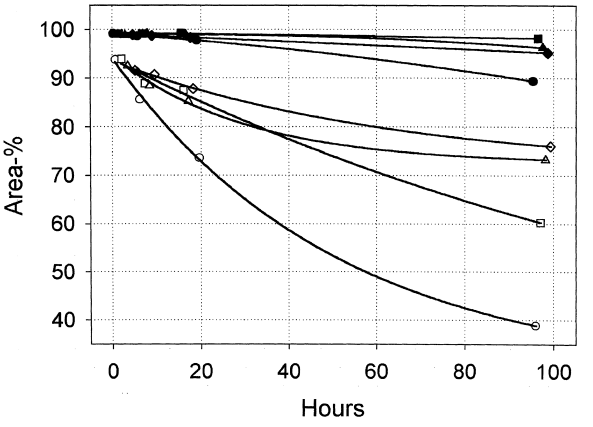

Fig. 4. The stability of solutions of pure CAR (94%) and pure CA (99%) is shown as a function of time and solvent. The solutions were stored in clear vials in the autosampler at room temperature. CAR in: (a) methanol (○, 260 μg/ml), (b) DMSO (□, 256 μg/ml), (c) DMSO–acetonitrile (10:90) (△, 285 μg/ml), ethyl acetate–acetonitrile (10:90) (◇, 245 μg/ml). CA in: (e) methanol (●, 237 μg/ml), (f) DMSO (■, 278 μg/ml), DMSO–acetonitrile (10:90) (▲, 247 μg/ml), (g) ethyl acetate–acetonitrile (10:90) (◆, 245 μg/ml).

spectrum is numerically very large to get a rugged method with regard to detection wavelength. Based on the RF-ratio figures above, the purity determination at 230 nm is less biased compared to 280 nm. The major impurities in the reference standards (isolated by preparative chromatography) are expected to be degradation products of the standard component, i.e. the impurities are closely related to the standard component and not of a completely different nature. The spectral properties are therefore expected to be similar to the standard component. This argument conforms to the observations made concerning the purity corrected response factor calculations of CAR, discussed in Section 3.4 and the recovery experiment in Section 3.5.

3.4. Stability of CAR and CA in solution

The stability of solutions of pure CAR and pure CA is dependent on the solvents used. Fig. 4 illustrates the decay of CAR and CA peak area-percentage as a function of storage time and solvent.

3.4.1. CAR

Pure CAR (95%) in solution has generally a very poor stability. The use of primary CAR standards for quantitative determinations could therefore imply a practical problem in unattended overnight sequences of analyses. Standards freshly prepared in the daytime have degraded during storage in the autosampler and will give rise to erroneous response factors when analysed. A practical solution to this problem is suggested.

Starting from an almost pure CAR standard in solution it is possible to correct for the purity using the area-percentage method. Each time the standard is analysed, its purity is calculated and the original response factor is corrected accordingly. In this way, it is possible to obtain the same purity-corrected response factors from all standards in a long sequence of analyses.

An example: Six repeated runs of a CAR standard, covering 30 h of analyses resulted in a purity-corrected average response factor with a relative standard deviation, RSD of 0.7% ($n=6$). Due to the poor stability of the CAR solution, the same six standard runs had CAR peak area-percentages (purities) ranging from 95% (0 h) to 75% (30 h). All data were obtained at 230 nm. This example supports the assumption that the degradation products have spectral properties similar to CAR (Section 3.3). Furthermore, it clearly demonstrates the necessity for adjusting the original response factors to obtain robust calibration data from standards, which is not freshly dissolved. This approach is practical for all

reference components provided they are prepared in separate solutions. Mixing more reference components in the same solution eliminates the possibility of calculating the individual purities.

As access to primary reference materials is limited, the introduction of a secondary standard is an alternative. Commercial rosemary extracts of known composition (calibrated against primary standards) can be used. Additionally, it is important to note that CAR and CA, present in rosemary extract solutions, are quite stable, which is documented in the recovery experiments discussed below in Section 3.5.

3.4.2. CA

The stability of CA in DMSO is good. The amount of CA in the solution is virtually unchanged after 95 h of storage in the autosampler. The same is not true for CA dissolved in methanol. The amount of CA decreased significantly within the first day of storage (Fig. 4). Richheimer et al. [5] used ''reagent alcohol'' (ethanol–methanol–isopropanol, 90:5:5) with small amounts of EDTA and phosphoric acid. CA dissolved in this solvent is stable for at least 24 h. Three solutions of the same standard were prepared and analysed during a 24-h period. The average peak area percentage of CA was 98.3%, with an RSD-value of 0.2% ($n=21$).

3.4.3. Clear vials vs. amber vials

The stability of CA solutions seems to be very dependent on the type of containers used for the solutions. Two types of sample vials, clear glass and amber glass were tested. Amber vials were expected to protect the solution from energy rich light, i.e. the stability was expected to be better in amber vials compared to clear vials. The sample vials were stored in the autosampler and not protected from light in other ways. However, a solution of CA in methanol (293 μg/ml) is less stable when stored in amber glass vials (Fig. 5). This observation indicated that amber glass was able to catalyse the oxidation process and the suspicion turned to transition metal ions in the glassware. The supplier of the glass vials informed that the amber glass had a significantly higher content of iron and titanium compared to clear glass. Expressed as percentage of oxide form: Fe_2O_3 (0.7–1%), TiO_2 (3–5%) in amber glass, while only a small amount of iron is present in clear glass: Fe_2O_3 (0.04%). The use of amber glassware should therefore be avoided as it appears that even the minute amounts of Fe and Ti ions on the surface of the glassware is able to accelerate the degradation of CA in solution.

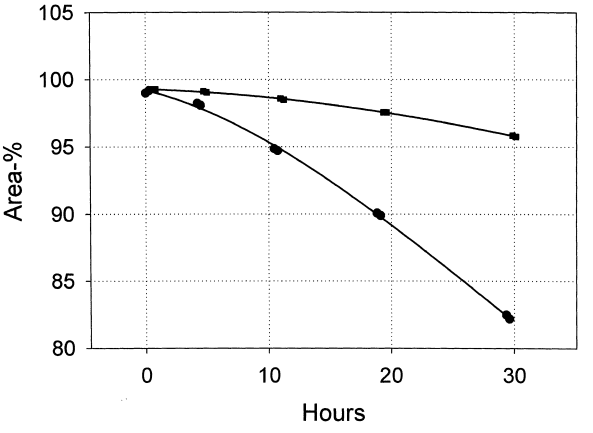

Fig. 5. Stability of CA dissolved in methanol and stored in clear vials (■) and amber vials (●), respectively.

3.5. Recovery of CAR and CA

The recovery of CAR and CA from rosemary extract was estimated by two parallel standard addition experiments—one with CAR and one with CA. A sample of rosemary extract was spiked at three concentration levels by dissolving in DMSO, adding an appropriate amount of standard solution, and diluting to volume with ACN. The amounts of standard added corresponded to approximately 25, 50 and 75%, respectively, of the expected amount present in the non-spiked sample. All samples were prepared at least in duplicate.

The purity-corrected response factors for CAR and CA were established from pure reference materials of CAR and CA, respectively. Applying these response factors, the observed peak areas were converted to observed amounts. The linear regression curves of the observed amounts against the added amounts were established for CAR and CA, respectively. The slopes of these regression curves are estimates of recoveries of CAR and CA. The recoveries are high: CAR 99.6% and CA 96.3% with standard errors of 1.7 and 1.6%, respectively (Fig. 6).

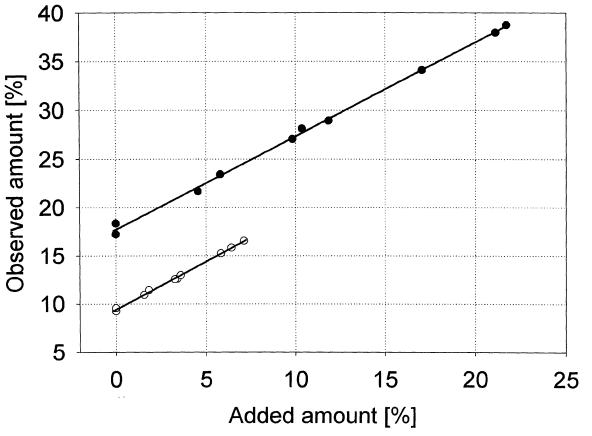

Fig. 6. Recovery of carnosol (○) and carnosic acid (●) from spiked rosemary extract; regression curves of added amounts (%) vs. observed amounts (%). The slopes, which are the estimated recoveries, are 0.996 and 0.963 for CAR and CA, respectively. The determination coefficients (R^2) for CAR and CA are 0.9976 and 0.9979, respectively. See Experimental section for conditions.

The high recoveries obtained documents the stability of the solutions. The two spiking series consisted of 10 samples each and covered a time interval of 21 h. All solutions are made at the same time, placed in the autosampler, and analysed overnight in a random sequence. If significant degradation took place during the sequence, it would be impossible to obtain recoveries close to 100%.

4. Conclusion

The present study discusses some practical challenges associated with accurate quantification of potentially labile antioxidants in rosemary extracts, namely CA and CAR.

CA dissolved in DMSO has very good stability. The same is not true for CAR. The stability of CAR in the solvents tested was very limited. However, CAR present in solutions of rosemary extracts is very stable. This is documented by a high recovery of CAR from rosemary extracts spiked with CAR. The recovery experiment covered a time span of 21 h and the recovery was 99.6%. In a parallel experiment, the recovery of CA was 96.3%. The purities of standard solutions are estimated as the peak area-percentage measured at 230 nm. Correcting for the measured purity of a calibration standard, a robust calibration/response factor can be established. This is important for automated overnight analytical sequences, where calibration solutions degrade during storage in the autosampler. The relative response of CAR compared to CA has been estimated to 0.92 (230 nm) and 1.36 (280 nm.). This factor must be applied to get an accurate estimate of the CAR content, when only CA is available as primary standard. Finally, the use of amber glass containers to protect solutions from light should be avoided due to the high content of metal ions (Fe, Ti) in the glassware. These metal ions seem to catalyse the autoxidation of CAR/CA in solution.

References

[1] A.G. Gonzales, C.M.L.J.G. Rodriguez, J. Chem. Res. S (1988) 114.
[2] K. Schwarz, W. Ternes, Z. Lebensm. Unters. Forsch. 195 (1992) 99.
[3] E. Wenkert, A. Fuchs, J.D. McChesney, J. Org. Chem. 30 (1965) 2931.
[4] R. Inatani, N. Nakatani, H. Fuwa, Agric. Biol. Chem. 47 (1983) 521.
[5] S.L. Richheimer, M.W. Bernart, G.A. King, M.C. Kent, D.T. Bailey, J. Am. Oil Chem. Soc. 73 (1996) 507.
[6] D. Bauman, M. Hadolin, A. Rizner-Hras, Z. Knez, Acta Aliment. 28 (1999) 15.
[7] S. Munné-Bosch, L. Alegre, K. Schwarz, Eur. Food Res. Technol. 210 (2000) 263.
[8] M.T. Tena, M. Valcárcel, P.J. Hidalgo, J.L. Ubera, Anal. Chem. 69 (1997) 521.
[9] K. Schwarz, W. Ternes, Z. Lebensm. Unters. Forsch. 195 (1992) 95.
[10] N. Okamura, Y. Fujimoto, S. Kuwabara, A. Yagi, J. Chromatogr. A 679 (1994) 381.
[11] J.-P. Yuan, H. Chen, F. Chen, J. Agric. Food Chem. 46 (1998) 2651.
[12] M.-E. Cuvelier, C. Berset, H. Richard, J. Agric. Food Chem. 42 (1994) 665.
[13] C. Bicchi, A. Binello, P. Rubiolo, Phytochem. Anal. 11 (2000) 236.