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Spectrophotometric methods to differentiate beers and evaluate beer ageing

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Abstract

The methodology most widely used to detect the antioxidant capacity (indirectly by $ABTS^{\cdot+}$ discoloration) has been modified to be applied to beer samples due to signal instabilities. A new and direct method to follow beer ageing has been developed based on a simple organic solvent extraction follow by molecular absorption spectrophotometry UV–VIS (MAS) measurement. Polyphenols and Maillard reaction products (MRPs) implied in beer ageing have been identified in the organic extract. A wide sampling of beers has been performed considering different countries, raw materials and brewing process. The two methods mentioned have been applied to the samples as well as the determination of total polyphenols, iron, copper and manganese by its role in ageing and in fermentation. In all cases, the data were obtained by triplicates. High correlation coefficients have been obtained between the $ABTS^{\cdot+}$ and the extraction method, therefore, this second method is proposed to differentiate antioxidant capacity and to follow beer ageing. The analysis of variance (ANOVA) has shown the possibility to differentiate types of beers and ecological from non-ecological beers.

Keywords: Beer stability; UV–VIS molecular absorption spectrophotometry; Polyphenols; Maillard reaction products; Statistical analysis

1. Introduction

Some food components have been recognized as protective agents in epidemiological studies in addition to their nutritional and sensorial properties. This is the case of natural antioxidants content in food. Many antioxidants can be lost as a consequence of food processing as well as during storage (Nicoli, Anese, Parpinel, Franceschi, & Lerici, 1997). For this reason, in some cases addition of antioxidants to the products prior to packaging is carried out (Walters, Hughes, & Bamforth, 1996).

The brewing industry is concerned with the stability of its final products. During storage, beer quality is gradually decreased and the production of stale flavour, the formation of haze, and browning occur. The speed of ageing depends on the storage conditions and the composition of beer. It is known that the development of certain carbonyl compounds plays an essential role in the loss of taste stability. A considerable number of phenolic compounds may act as antioxidants, with mechanism involving both free radical scavenging and metal chelation (Fantozzi et al., 1998). On the other hand, the role of phenols as antioxidants is controversial. In fact, contradicting results exists on the effectiveness of some phenolic compounds as potential antioxidants pointing out the sulphite as a relevant antioxidant in beer (Andersen, Outtrup, & Skibsted, 2000; Forster et al., 1999). The polyphenols during oxidation can polymerize forming tannins, which are able to react with proteins forming insoluble compounds, which are known as haze. So the polyphenols are to a great extent

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responsible for the colloidal stability of beer (Andersen et al., 2000; Mc Murrough, Kelly, & Byrne, 1992; Siebert, 1999; Sobiech, Neumann, & Wabner, 1998). It is well known that the Maillard reaction products (MRPs) may also influence the oxidative stability and shelf life of several foods. Furthermore, they are effective synergists in combination with phenolic antioxidants. Naturally occurring antioxidants could be significantly lost as a consequence of processing and storage, but antioxidant MRPs are formed (Morales & Jiménez-Pérez, 2001). There is accumulating evidence for the ability of MRPs to complex metal ions (O'Brien & Morryssey, 1997).

Several methods have been developed to evaluate global antioxidant capacity, they are usually based on the evaluation of the free radical scavenging capacity. The most commonly used are based on molecular absorption spectrophotometry UV–VIS (MAS) due to its simplicity to handle and low cost. They are indirect methods, based on the pre-formation of a free radical from an aromatic organic compound, such as 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Re et al., 1999), the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Kaneda, Kobayashi, Furusho, Sahara, & Koshino, 1995) and the free radical *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD) (Fogliano, Verde, Randazzo, & Ritieni, 1999). Other methods based on electrochemical techniques (Sobiech et al., 1998) and on the determination of the lag time by electron spin resonance (ESR) (Uchida & Ono, 1996; Uchida, Suga, & Ono, 1996) have been used.

In this paper, a simple and direct method to differentiate beers and to evaluate ageing is presented, based on an extraction from beer with an organic solvent and a measurement by MAS. The new proposed method shows high correlation with the ABTS method, modified by us to be applied to beer samples. Some trace metals related to stability and brewing process are determined. Statistical techniques, applied to a wide sampling of beers, show the capability to use this extraction method as index for the brewing industry to evaluate the stability and ageing of beers.

2. Materials and methods

2.1. Reagents

Ethyl methyl ketone (EMK; Merck, Damstadt, Germany), 4-methyl-2-pentanone (MIBK; Panreac, Barcelona, Spain), cyclohexane (Panreac, Barcelona, Spain), sodium sulphate anhydre (Panreac, Damstadt, Germany), 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma, Steinheim, Germany), glycine-hydrochloride (Sigma, Steinheim, Germany), peroxidase from Horseradish highly stabilized (HRP; Sigma, Steinheim, Germany), hydrogen peroxide 30% (Merck, Damstadt, Germany), Folin–Ciocalteu's reagents (Panreac, Barcelona, Spain), sodium carbonate anhydre (Panreac, Barcelona, Spain), nitric acid 65% (Merck, Damstadt, Germany), ICP multi-element standard solution IV (Merck, Damstadt, Germany) and Milli-Q water were used.

2.2. Instrumentation

Spectrophotometer (Philips PU 8750), microwave oven (CEM MDS.2000) equipped with Teflon vessels (PFA, 120 ml, 220 psi) and pressure regulator, cryogenic bath (HAAKE E 1), ultrasonic bath (Selecta), ultrasonic liquid processor, oven (Heraeus), mechanic agitation system were used.

2.3. Sampling

The samples were obtained from a local store. In the previous test we used 6 lager beers (Budweiser, Cruzcampo, Alhambra, Damm, Newcastle and Pilsner) and 3 stout beers (Mahou, Leffe and Negra Modelo). For the statistical study the analyzed beers were: Heineken, Carlsberg, Cruzcampo, Alhambra, Stock Premiere, Speciale Flag, Flag Pils (lagers, cans). Bavaria, Alhambra, Laiker, San Miguel (lagers, cans, alcohol free). Budějovick, Pilsner Urquell, Dos equis (lagers, bottle, additives free). Carlsberg, Heineken, Cruzcampo, Alhambra reserve, Damm (lagers, bottle). San Miguel, The 4 elements, Škokrone, Neumarkter lammsbrëu (lagers, bottle, ecological). Guinness, Mahou, Primátor, Negra modelo (stout, bottle). Neumarkter lammsbrëu (stout, bottle, ecological). Paulauer, Erdinger, Spital, Franziskaner (weizen wheat, bottle). Diät Pils (lager, can).

2.4. Liquid–liquid extraction and spectrophotometric measurement

The beer samples were previously degasified by means of ultrasounds to be able to take an exact beer volume. Twenty millilitres of fresh beer were extracted with 20 mL EMK after 5 min shaking by mechanic agitation system. This phase was measured by molecular absorption spectrophotometry and a broad band was obtained with a maximum at $\lambda = 333$ nm.

2.5. Determination of antioxidant capacity

The $ABTS^{\bullet+}$ was prepared by enzymatic oxidation of ABTS with H_2O_2 and peroxidase (Millar, Rice-Evans, Davies, Gopinathan, & Milner, 1993). The reagents concentrations were 60 μM of ABTS, 50 μM of H_2O_2, 0.25 nM of peroxidase and 50 mM of glycine-HCl (Arnao, Cano, Hernández-Ruiz, García-Cánovas, & Acosta, 1996). The procedure is the following: 100 μL of

distilled water are added to 2.5 mL of $ABTS^{\bullet+}$ and the absorbance is measured at $\lambda = 414$ nm. After that, 100 μL of beer is added into the spectrophotometer cuvette that contains 2.5 mL of $ABTS^{\bullet+}$ and the new absorbance is immediately measured. The absorbance difference is written down.

2.6. Measurement of the total polyphenolic: Folin–Ciocalteu method (Singleton & Rossi, 1965)

Half a millilitre of beer, 30 mL of distilled water, 2.5 mL of Folin–Ciocalteu reagent, 7.5 mL of a solution of sodium carbonate anhydrous at 20% and distilled water to make up the total volume of 50 mL. The solution is shaken to homogenize it and after 2 h the absorbance at 765 nm is measured, using a blank prepared with distilled water.

2.7. Metal determination by flame atomic absorption spectrometry (Bellido-Milla, Moreno-Pérez, & Hernández-Artiga, 2000)

Beer samples were digested in the microwave oven before of the determination of metals by atomic absorption spectrometry (AAS). The digestion procedure is described for three perfluoroalcoxy Teflon vessels in a microwave oven. A program with two stages was used. In the first stage, 20 mL of degassed beer was placed in each vessels and 3 mL of HNO_3 was added. The vessels were closed, and a three-step programme was set, each step at 40% power, 15 psi maximum pressure and duration of 2 min. The vessels were left to cool in a cryogenic bath at 5 °C for 5 min. The reactors were opened, and in a second stage 2 mL of 30% H_2O_2 was added, the setting was 40% power, 20 psi maximum pressure, and 3 min. After cooling at room temperature, the vessels were opened, and the product was transferred to a 25 mL volumetric flask and diluted to volume with Milli-Q water. Fe, Cu and Mn were determined by AAS in the digested samples. An air-acetylene flame was used for the three metals.

2.8. Statistical analysis

The statistical analysis was performed with the computer software Statgraphics Plus 5.1.

3. Results and discussion

3.1. Organic solvent extraction and UV–VIS molecular absorption spectrophotometry

The responsible compounds for beer stability exhibit a characteristic absorption in the UV region from 240 to 310 nm. The integral absorption for this region has been successfully used as an easy to measure sum parameter for characterizing the ageing status of beer (Varmuza, Steiner, Glinsner, & Klein, 2002). The absorption at the maximum exhibited at 269 nm (Bellido-Milla et al., 2000) together with the metallic content has been used to classify samples into different groups.

The MAS combined with a previous extraction has been used by other authors to study the residual organic content of biological digests (Würfels, Jackwerth, & Stoeppler, 1989) and by us to evaluate the organic content of beer digests (Bellido-Milla, Oñate-Jaén, Palacios-Santander, Palacios-Tejero, & Hernández-Artiga, 2004) with the aim to determine trace metals. In both cases the 4-methyl-2-pentanone (MIBK) has been used to carry out the extraction. On the other hand, beers develop compounds that end up producing haze since the colloidal stability is broken down; therefore, the extraction with a solvent of low polarity can isolate these compounds. In our case, we thought of the possibility to use the absorption maximum at 333 nm of the extract with the aim to characterize, differentiate or detect the ageing state of fresh beer.

In previous tests, MIBK was used for an extraction of fresh beer. The phase separation was not good and Na_2SO_4 was needed to dry the organic phase. Nevertheless, a broad band was obtained with a maximum at $\lambda = 333$ nm similar to the one obtained for the above-mentioned beer digests. To improve the extraction process other organic solvents were tried. Repeatability and sensitivity tests were performed with MIBK, EMK and cyclohexane for several beers.

Mahou (stout) in bottle, Budweiser (lager) in bottle and one can of Cruzcampo (lager) were submitted to the extraction process. The beer samples were previously degasified by means of ultrasounds to be able to take an exact beer volume. The results obtained are shown in Table 1.

The separation phase process for EMK apparently was the best; in the other two cases emulsions in the interphase were observed. On the other hand, this solvent shows more sensitivity. The extract absorbance was stable at least a week. Therefore, the EMK was

Table 1
Absorbance obtained at λ_{max} after extraction

Beer type	MIBK (333 nm)	EMK (333 nm)	Cyclohexane (278 nm)
Mahou (stout)	1.172 (1.70)	2.199 (0.80)	1.540 (14.86)
Budweiser (lager)	0.361 (0.69)	0.649 (1.82)	0.413 (14.89)
Cruzcampo (lager)	0.423 (3.03)	0.808 (4.35)	1.098 (9.99)

The results are the mean of three replicates. The relative standard deviation (%) is shown in brackets.

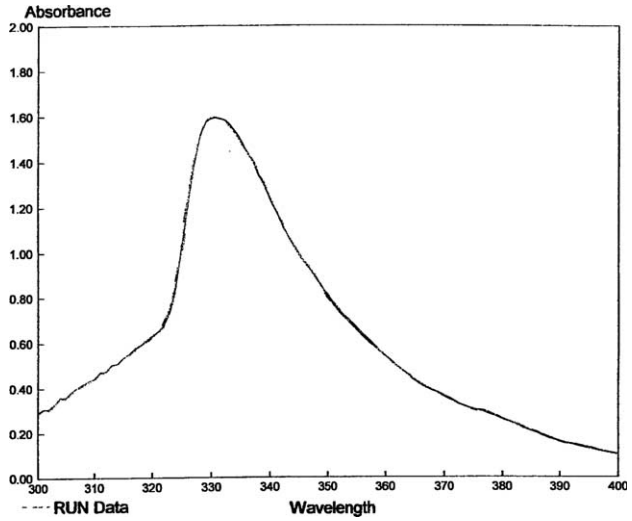

Fig. 1. Molecular absorption spectrum UV–VIS of EMK extract for Pilsner beer.

chosen to perform the extraction. In Fig. 1 the extract spectrum is shown with a maximum at $\lambda = 331$ nm. In the other cases the spectrum obtained was qualitatively similar.

In order to evaluate the possibilities to differentiate beers with an EMK extraction, seven beer types were tested by triplicate: Mahou (stout), Budweiser, Cruzcampo, Pilsner, Damm, Alhambra and Newscastle (lagers). The absorbance of the EMK extracts were measured. An analysis of variance (ANOVA) was used to determine statistically significant differences among arithmetical means of the triplicates. The results obtained were $F = 719.57$ and $p_{\text{value}} = 0.000$. Since the p-value of the F test is <0.05, there are significant differences among the mean absorbances of different types of beers with a confidence level of 95%.

We can find in the bibliography references (Bellido-Milla et al., 2004; Maillard, Soum, Boivin, & Berset, 1996) that can be used to identify the groups of compounds extracted with EMK that show a band with a maximum ~333 nm. A method has been described to estimate the levels of total phenols and the main groups of phenols in barley and malt by MAS (Maillard et al., 1996). Thin layer chromatography shows the presence of three main phenolic groups. Several spectrophotometric tests with standards corresponding to these three phenolic groups showed that the hydroxicinnamic derivatives exhibit a band with a maximum around 330 nm very similar to the one obtained by us with the EMK extract. Therefore, we can suppose the presence of this phenolic group in our extract. On the other hand, in a previous work developed by us (Bellido-Milla et al., 2004), phenolic acids were detected in beers by MAS with a previous extraction with MIBK and by high pressure liquid chromatography (HPLC) with photodiode array detector in both cases at 333 nm.

Phenolic compounds are not the only constituents responsible for the antioxidant activity in beers. MRPs or intermediates have also been reported as antioxidant components (Morales & Jiménez-Pérez, 2001). Some of them will probably go into the EMK extract. The Maillard reaction development is generally measured by the absorbance increase either at 294 nm (early MRPs), 320–350 (soluble premelanoidins) or 420–450 (advanced MRPs) nm. UV–VIS spectra of HPLC studies with diode array detector shows also a peak at 334.4 nm corresponding probably to MRPs of low polarity (premelanoidins) (Billaud, Brun-Mérimée, Louarme, & Nicolas, 2004). A test was carried out to study the influence of heating on the absorbance of the organic extract since MRPs are formed when heating between 70–110 °C. When beer samples are heated at 75 °C they become darker and solid species are formed. The changes produced are different to the ones obtained with ageing, and these treatment is not valid to simulate a natural ageing. Therefore, heating at 75 °C can be used to try to identify the compounds extracted with EMK. With this aim, Cruzcampo and Heineken beers were extracted and measured. The absorbances obtained at 333 nm were 1.071 and 0.880, respectively. After that, they were heated at 75 °C during three days, an extraction with EMK was performed and the absorbance measured at 333 nm. The results obtained were 1.150 and 1.016. We confirm with this absorbance increment that the EMK extract contains MRPs as suspected with the data found in the bibliography (Billaud et al., 2004).

With the aim to confirm that the EMK extract contained antioxidants, the antioxidant capacity of some beers was measured before and after solvent extraction (by the MAS method described afterwards). The results obtained before extraction were: Absorbance for Cruzcampo (lager) 0.603, for Mahou (stout) 1.055 and Carlsberg (lager) 0.564. This was compared with antioxidant capacity of the aqueous phase remaining after the extraction. The results were: Cruzcampo 0.418, Mahou 0.795 and Carlsberg 0.372. Since this second measurement was lower, we conclude that we extract antioxidant species.

3.2. Antioxidant capacity by decolouration of the $ABTS^{\cdot+}$

Beers contain compounds with antioxidant properties such as reducing sugars, phenolic compounds, vitamins and MRPs. It is possible to measure individually these groups of compounds but this methodology may not accurately reflect their combined action and the measurement of the total antioxidant activity is considered an important food property. Recently, the ABTS method (Millar et al., 1993) is presented as an excellent tool for determining the antioxidant activity of plant material and beverages (Arnao et al., 1996; Cano, Hernández-Ruiz, García-Cánovas, Acosta, & Arnao, 1998;

Cano-Lario, Acosta-Echevarrıa, & Bañón-Arnao, 1998).

The $ABTS^{\cdot+}$ was prepared by enzymatic oxidation of ABTS with H_2O_2 and peroxidase (end point method). Some previous tests with different beers (Carlsberg, Pilsner, Leffe and Negra Modelo) showed qualitatively similar results and they can be described as follows: The first absorbance decrease is not stable and the absorbance continues slowly decreasing. After 30 min, another decrease of similar magnitude had taken place and after that, the absorbance decrease shows a clear tendency to stabilize. The precision was calculated measuring triplicates of these beers. The RSD found were between 0.3 and 8.74. This first and quick decrease followed by a slow and continuous decrease is not described in the literature. This is shown in Fig. 2 for the beers studied.

The correlation coefficient between the absorbance decrease at $t = 0$ (measured immediately after mixing) and the absorbance decrease at $t = 90$ min was $r = 0.938$ (Fig. 3), therefore, we can take the absorbance at $t = 0$ as a differentiate property of beers.

To ensure the capability to differentiate, seven different beers were tested and the first absorbance decrease (A) was measured. The individual beers showed different values as can be observed in Table 2. The data were obtained by triplicate. An analysis of variance (ANOVA) was used to determine statistically significant differences for a confidence level of 95%. The results obtained were $F = 457.25$ and $p_{value} = 0.000$. Tukey homogeneity test was applied to establish these differences. The results indicate that all beers show statistically significant differences except Budweise that can be confounded with Alhambra and Newcastle with Cruzcampo.

With these results we thought that the measurement of the immediate absorbance decrease could be an interesting data since it represents the group of antioxidants with a rapid mechanism when free radicals are formed naturally in this beverage. The slow antioxidants are not so interesting since a slow radical scavenger reaction allows the oxidation of the beer compounds.

Other strategy with the ABTS has been described in the literature known as "lag time". This is based on the addition of the antioxidant to the ABTS/peroxidase/H_2O_2 system to retard the formation of the $ABTS^{\cdot+}$ (Arnao et al., 1996). It is a longer and more complicated method than the end-point method modified by us. Besides, the generation of radicals before the antioxidants are added prevents interference of compounds, which affect radical formation (Van den Berg, Haenen, Van den Berg, & Bast, 1999). Some authors perform a quantitative evaluation of antioxidant capacity using ascorbic acid or trolox (Arnao et al., 1996; Cano et al., 1998) but other authors believe that this methodology is troublesome and inadequate, but, in any case, it can be used to provide a ranking order of antioxidant capacity (Van

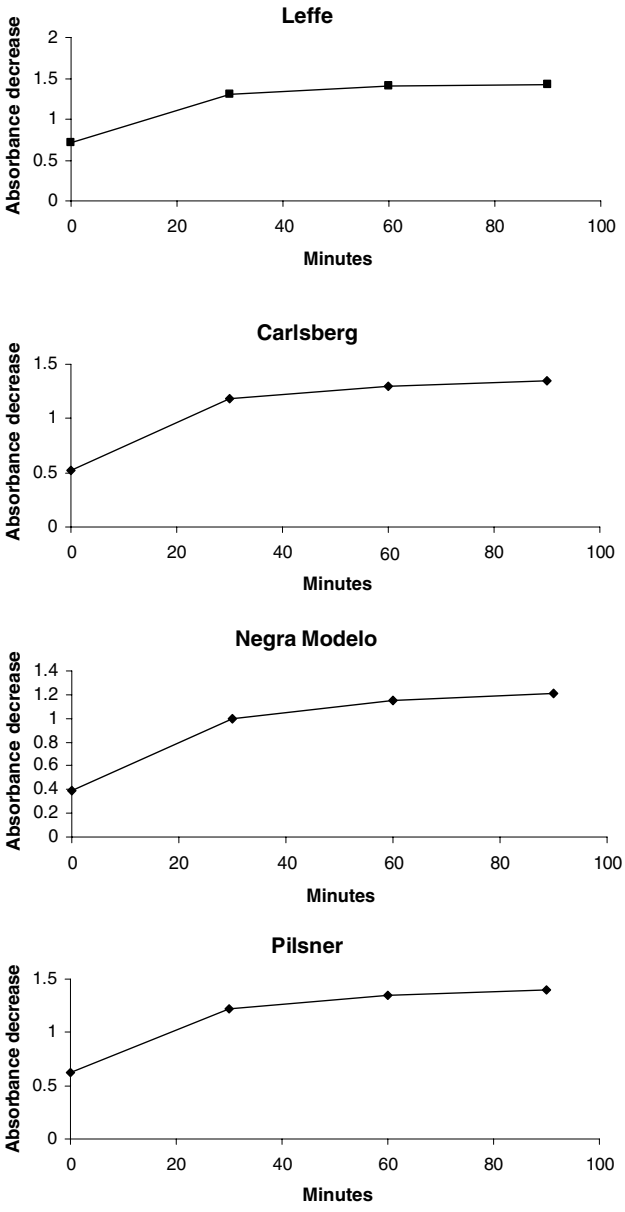

Fig. 2. $ABTS^{\cdot+}$ absorbance decrease vs. time (minutes) for different types of beers.

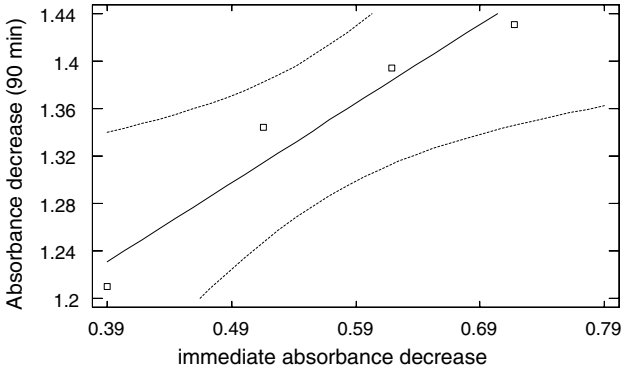

Fig. 3. Linear regression model for absorbance decrease at 90 min vs. immediate absorbance decrease.

Table 2
Absorbance decrease obtained by the modified $ABTS^{\cdot+}$ method

Beers	$ABTS^{\cdot+}$ absorbance decrease (A)	RSD (%)
Mahou	1.090	0.47
Cruzcampo	1.026	2.10
Budweiser	0.639	1.02
Alhambra	0.765	1.31
Damm	0.969	1.08
Newscastle	0.866	1.00
Pilsner	0.996	1.87

The results are the mean of the three replicates.

den Berg et al., 1999). We think that the immediate $ABTS^{\cdot+}$ absorbance decrease, provoked by the different type of beers proposed by us provide also a ranking of antioxidant capacity.

Therefore, we proposed this rapid and precise methodology as an index to compare the antioxidant capacity of beers that really is related to the taste and stability of this beverage, since it represents the antioxidants of rapid kinetic.

3.3. Extraction with organic solvent and antioxidant capacity as indexes to follow beer ageing

During storage, beer quality is gradually decreased and the production of stale flavour, the formation of haze and browning occur.

Many researchers have investigated beer flavour stability. Most of them have focused on techniques for determining a compound or a small group of similar compounds. For instance volatile aldehydes (Hashimoto & Eshima, 1977), E-2-nonenal (Guido, Fortunato, Rodrigues, & Barros, 2003), β-damascenone (Guido et al., 2003), furfural, 5-hydroxymethylfurfural (Varmuza et al., 2002). However, it seems that perceptible staling compounds might due to a complex mixture. The mechanism of beer staling has not been fully elucidated, an oxidative reaction of beer has been recognized as the most important cause of staling flavour development (Uchida & Ono, 1996).

Forcing ageing test at 60 °C has been proposed to save analytical time. Qualitatively similar results have been found comparing with natural ageing (Uchida & Ono, 1996) (when higher temperatures are used the changes undergone are not comparable to a natural ageing). With this aim, four types of beers were heated to 60 °C during 7 days and the same types were aged naturally during five weeks. The absorption of the EMK extract and the antioxidant capacity was measured in beers recently purchased and after natural and forced ageing. The results for the EMK extract and for the antioxidant capacity are shown in Table 3.

In all cases, the values observed diminish with ageing. The Pilsner beer shows in both ageing methods a higher decrease, probably due to the traditional brewing without additives. Therefore, both methodologies can be used to follow beer ageing. Other authors have observed a similar behaviour for natural and forced ageing (60°) when ESR (Uchida et al., 1996) is used. The decrease of absorbance for EMK extract can be due to the fact that some of the extracted species (mainly polyphenols) are transforming with ageing and they do not contribute to the absorbance signal.

3.4. Iron, copper and manganese and its relationship with organic matter

Iron and copper play an important role as catalyst in the oxidation of organic compounds that are responsible for the stability and flavour of beers. Manganese concentration has influence in beer fermentation rate (Bromberg et al., 1997). Previously, the authors based on trace metal contents, have carried out differentiation and classification of beers (Bellido-Milla et al., 2000). In that work, the total content of eight metals for 25 beers was determined by atomic spectroscopy. The results for Fe were between 0.06 and 0.55 mg/l, for Cu between 0.02 and 0.07 mg/l and for Mn between 0.06 and 0.21 mg/l. As it is known, Fe and Cu are able to form complex compounds with organic matter, but the Mn ability to form complexes is low. The capacity of metallic ions to be bound to phenolic acids is reported in the literature (Montanari, Perretti, Natella, Guidi, & Fantozzi, 1999). On the other hand, there is accumulating evidence for the ability of MRPs to complex metal ions. It may be hypotyzed that MRPs might bind metal ions more strongly than the amino acid from which they derived (O'Brien & Morryssey, 1997). The loss of antioxidant capacity of these organic compounds when they form metallic complexes have been reported (Irwin, Barker, & Pipasts, 1991; Wijewickreme & Kitts, 1998). All these reactions may contribute to

Table 3
Results of natural and forced ageing

Beers	EMK extract absorbance			Absorbance decrease of $ABTS^{\cdot+}$		
	Control beer	Natural ageing	Forced ageing	Control beer	Natural ageing	Forced ageing
Carlsberg	0.840	0.766	0.783	0.580	0.493	0.346
Pilsner	1.666	1.328	1.273	0.719	0.600	0.303
Leffe	1.707	1.526	1.673	0.814	0.517	0.623
Negra Modelo	0.963	1.025	0.938	0.552	0.394	0.293

changes during beer ageing and to final beer quality (Blanco, Caballero, Rojas, Gómez, & Álvarez, 2003).

Copper ion in beer is presumably bound to amino acids and proteins, but the nature and extent of the binding do not complete prevent participation of the copper in oxidation reaction. Melanoidins bind Fe and Cu very strongly and it has been suggested that this ability contributes to their antioxidant properties. Although not well documented, dark malts have been alluded to as producing more flavour-stable beers than pale malts. Perhaps, this effect can be ascribed to their higher melanoidin content and hence higher metal-binding capacity of dark malts (Irwin et al., 1991). In the same work, an obvious correlation was found between the stale flavour intensity and the copper concentration of 31 beers. No such correlation was observed with iron, suggesting that copper has a more deleterious effect on beer flavour instability.

The effect of traces of Cu and Fe in beer does not just depend on the total concentration of the metal but also on the type of compounds formed. The iron has a higher capacity to form stable compounds than copper (Bellido-Milla et al., 2004). Non-complexed Fe ions may have a negative effect on the beer quality whereas the complexed form will not. It has been found that when Fe is introduced after fermentation can cause haze because there is not sufficient complexing organic matter (Svendsen & Lund, 2000). The ability of MRPs and phenolic compounds to complex metal ions has been often described (Fantozzi et al., 1998; Montanari et al., 1999; O'Brien & Morryssey, 1997). The binding of hop acids to trivalent cations such as Fe (III) has been demonstrated and also the influence of iron-complexes in foam, colour and stability (Blanco et al., 2003).

3.5. Statistical analysis

In order to study the possibilities of the new methodologies developed in this work, 34 beers were analyzed. They are listed in Section 2. They were obtained from local stores and represent the types of beers readily available to consumers. The absorbance of EMK extract and the antioxidant capacity were determined as described above. Total polyphenols were also measured by MAS (Singleton & Rossi, 1965) by its potential role in the antioxidant capacity (Fantozzi et al., 1998; Montanari et al., 1999). These three variables were determined in fresh and aged beers (60 °C during a week). Fe, Cu and Mn were also determined by AAS after digestion in closed reactors assisted by microwaves (Bellido-Milla et al., 2000).

In Fig. 4, the histograms of the distribution for the variables are represented. All data were the means of triplicates. Fe and Mn exhibit a great span but Cu shows a small span since in most cases its concentration is

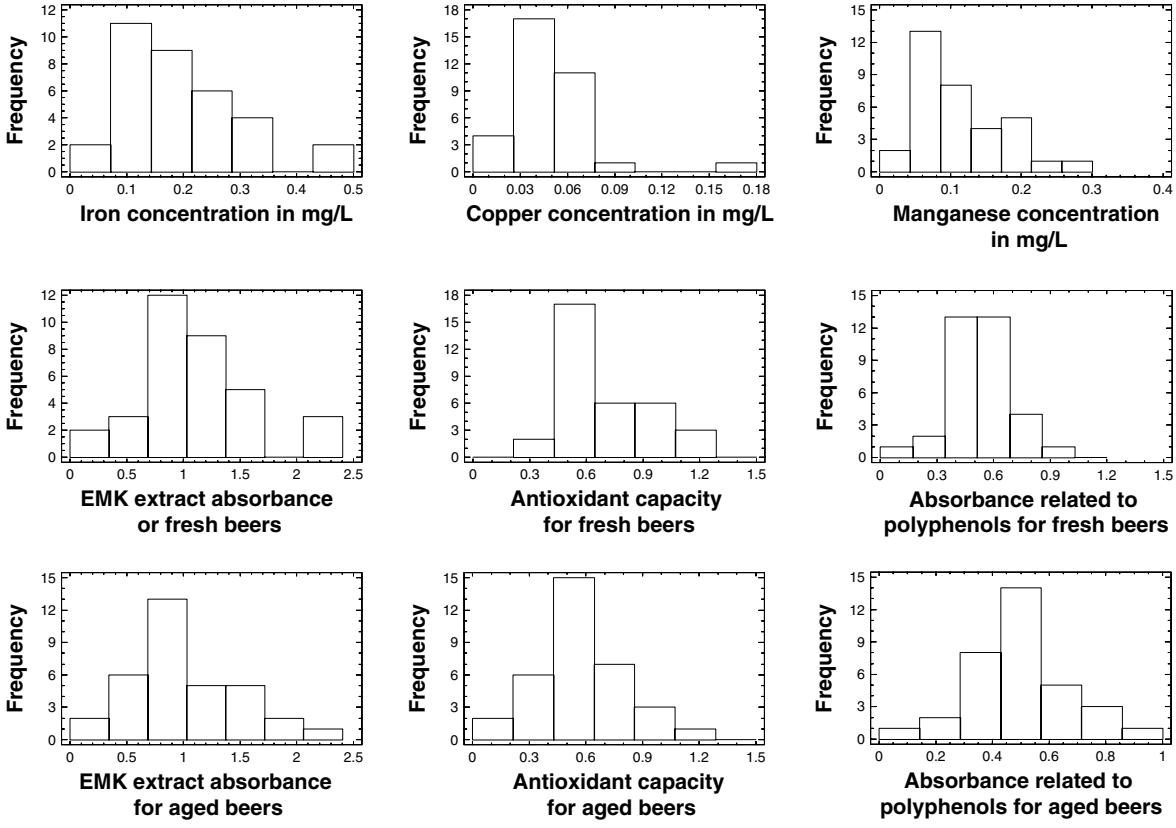

Fig. 4. Histograms of the variable distributions for 34 different beers.

subjected to legislation. The variables related to organic matter show also a great span, being the EMK extract absorbance the one exhibiting greater absorbance range. This fact implies a better possibility to differentiate beers.

The correlation coefficients (r) between the three variables related to beer stability EMK extract (E), antioxidant capacity (A) and total polyphenols (P) are the following: E vs. $P = 0.849$, P vs. $A = 0.866$ and E vs. $A = 0.896$ for the 34 fresh beers. They are high, above all in the case of absorbance extract vs. antioxidant capacity. This allows predicting the antioxidant capacity by means of the mathematical model $A = 0.234 + 0.424E$. Therefore, with a simple extraction and measurement by MAS the antioxidant capacity can be predicted avoiding the use of an organic reagent and the development of the corresponding free radical. It is shown in Fig. 5. The correlation coefficients between fresh (E, A and P) and aged beers (E', A' and P') for the three variables are: E vs. $E' = 0.995$, P vs. $P' = 0.992$ and A vs. $A' = 0.939$. Therefore, it is possible to predict the value of the aged beers from the values of the fresh beers in the three cases. Once again the EMK extract gives the highest correlation.

With the aim to find out whether a direct correlation exists between the endogenous Cu, Fe or Mn and the three organic variables, the corresponding correlation coefficients were calculated. The results obtained were in all cases very low. Therefore, a direct interaction between these three trace metals and the three organic variables has not been found due to the great amount of chemical reactions among the beer components.

The analysis of variance was tested to differentiate groups of beers by means of the organic variables or by the trace metals studied, for a confidence level of 95%. The three organic variables show statistically significant differences between ecological and non-ecological beers (antioxidant capacity: $F = 6.48$ and $p_{value} = 0.016$, polyphenols $F = 4.73$ and $p_{value} = 0.037$ and EMK extract $F = 4.74$ and $p_{value} = 0.0369$). These three organic variables are also able to differentiate types of beers. A, P and E differentiate lager from stout, besides E is also able to differentiate stout from weizen ($F = 8.60$ and $p_{value} = 0.0011$). This result is shown in Fig. 6 and they can be explained by differences in brewing process.

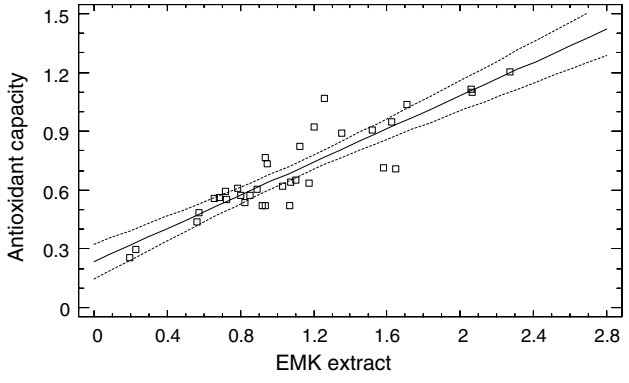

Fig. 5. Linear regression model for antioxidant capacity (A) vs. EMK extract absorbance (E).

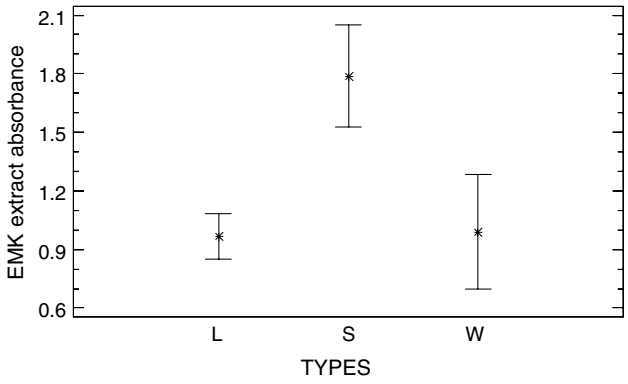

Fig. 6. Analysis of variance. Confidence intervals of the EMK extract absorbance for Lager (L), Stout (S) and Weizen (W).

Iron or copper content does not differentiate groups of beers in spite of the great variety of samples analysed. However, Mn is able to differentiate ecological from non-ecological beers ($F = 6.04$ and $p_{value} = 0.0196$) and Lager from Weizen beers ($F = 6.07$ and $p_{value} = 0.0060$) probably due to the different quality raw materials used in brewing.

4. Conclusion

A new rapid and simple index to follow beer ageing useful for quality control has been developed. The widely used $ABTS^{\cdot+}$ method to detect antioxidant capacity has been modified to be applied to beers. High correlations have been found between these two methodologies. Differentiation from ecological and non-ecological beers has been possible by means of the new index, by the modified antioxidant capacity method or by the total manganese content. Types of beers have also been differentiated by the new index, by the antioxidant capacity and by the Mn content.

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